JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

January 1962 volume 51, number 1

Review Article

ANTIBIOTICS 1956—1961

By ROBERTSON PRATT

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Received from the University of California Medical Center, San Francisco 22.

INTRODUCTION

ANTIBIOTIC is a young word in terms of the history of our language. It stems from antibiosis, a term coined in 1889 by Vuillemin (1) to describe a broad philosophic concept of a biologic relationship often observed but not previously specifically named. The term existed in the scientific vocabulary only a little more than 50 years before it was applied in the health professions in its present very restricted sense, and less than a decade later became a household word known to most well-informed laymen.

Rational application of the phenomenon of antibiosis for treatment of diseases according to scientific principles is a development of the last 20 years, but crude antibiotic therapy was practiced empirically at least 2,500 years ago and there was a prolonged flurry of laboratory experiments and less well-controlled clinical experimentation which started more than 80 years ago with the work of Pasteur and his students on anthrax (2) and was pursued enthusiastically, if not always carefully, by others in a wide array of infectious diseases for nearly 40 years. An antibiotic product, pyocyanase, derived from *Pseudomonas pyocyanea* (*aeruginosa*) was marketed in Europe before World War I. These early historical developments have been reviewed (3, 4).

The current era of antibiotic therapy began in 1929 with the now celebrated observation of Fleming and his coining the term "*penicillin*" to designate the then unidentified antibacterial metabolic product of the mold (5). This was followed by a lag period of a little more than a decade before the logarithmic period in which we now are emerged.

The antibiotics discussed below have been selected from those discovered or first marketed since 1955. Thus, the subjects reviewed represent some of the developments of approximately the most recent quarter of the active period of the current era of antibiotic therapy.

The discussion of specific antibiotics is preceded by some general comments on topics of current interest and import in antibiotic therapy. In general, these are topics the significance of which has been widely recognized only since 1955, although a few voices in the wilderness were warning about some of them prior to that time.

GENERAL CONSIDERATIONS

Blood Levels.—It is convenient and it has become conventional to compare different antibiotics or different formulations of a given antibiotic in terms of the concentration of the drug demonstrable in the serum at different times after a single oral dose or during a course of multiple doses. Unfortunately, there is a natural tendency to assume that such comparisons afford a valid basis for predicting therapeutic efficacy of the products, and much of the advertising of antibiotic products has taken advantage of this tendency. While comparison of blood levels produced may lead to valid conclusions regarding the relative therapeutic efficacy of different formulations of the same antibiotic, it is obvious that it is not equally justifiable to use such figures in the same way for evaluating different antibiotics. The data may provide useful physiologic and pharmacologic information about the drugs, e.g., absorption, resorption, excretion, metabolic degradation, etc., but they do not necessarily indicate anything about relative therapeutic efficacy.

First, there is the matter of bacterial sensitivity. A low concentration of a drug to which the infecting pathogen is very sensitive may be expected to be more effective than a considerably higher concentration of one to which it is only moderately sensitive.

Second, bacteria are more common in other tissues than in blood. Therefore, since the important point for therapeutics is the activity of the drug against the specific organism and the concentration of the drug at the site of infection where the organisms are, the blood level *per se* has little real significance in evaluation of therapeutic potential unless the gradient between the blood and the different tissues is known so that the concentration of the drug at the actual sites of infection can be calculated. Indeed, a study of "distribution volumes" of several antibiotics, including penicillins G and V; the tetracyclines; the macrolides, erythromycin and oleandomycin; streptomycin; and novobiocin, indicated that "high blood levels result in a low distribution volume, and vice versa" (6).

Probably more useful than serum concentration for evaluating potential therapeutic value of an antibiotic is the serum dilution factor, i.e., the extent to which the serum can be diluted and still remain bactericidal or bacteriostatic. Although this factor does not indicate anything about the concentration of the drug at the site of infection, it does at least take cognizance of the sensitivity of the organism to the specific antibiotic under investigation.

Enhancement of Blood and/or Lymph Levels.—Following the general line of fallacious reasoning referred to above, there has been considerable emphasis on preparations

formulated with agents reputed to provide higher blood levels of the antibiotic than can normally be achieved with equivalent doses of similar preparations lacking the adjuvant. The emphasis has been greatest in the tetracycline field where, it has been stated, "economic competition among pharmaceutical manufacturers for markets and over-zealous advertising agencies have led to some extravagant claims regarding enhancement of blood levels attainable with various formulations of tetracyclines" (7).

Careful experimentation and objectively critical appraisal of published results indicate that many of the claims of enhanced blood levels achieved with tetracyclines formulated with glucosamine, sodium hexametaphosphate, and other "potentiating" agents cannot be statistically supported and that for all practical purposes the various formulations are comparable in clinical efficacy whether or not they contain the alleged blood-level-enhancing factors. Indeed, most of the work reported prior to October 1957 on enhancement can be ignored as erroneous and irrelevant because results produced with the new formulations were compared to those produced with the then regularly available commercial formulations which contained calcium and/or magnesium salts that chelate tetracyclines, thus reducing their availability for absorption. Therefore, most of the results before late 1957 showing improved blood levels with the new formulations were due more to the depressing action of additives in the control products than to enhancing agents in the experimental ones. Moreover, many reports were based on studies employing single oral doses given to fasting, normal, healthy volunteers, an experimental situation bearing little resemblance to actual clinical practice with patients who receive multiple doses at different times without regard to time elapsed since the last meal. Subsequent work has failed to reveal significant, if any, advantages of the "enhanced" products under practical clinical conditions. For further discussion of the controversy over blood levels of tetracyclines and other antibiotic preparations, see reference 7 (pp. 62 and 214) and references 8–15.

Related to the blood-level *vs.* tissue-level controversy and the emphasis on agents to enhance blood levels is the matter of antibiotic concentrations in the lymph. The importance of the lymphatic system in bacterial invasion suggests the desirability of supporting this protective mechanism by providing significant concentrations of appropriate antibiotics in the lymph during bacterial attack. Macromolecular salts of various antibiotic bases with appropriate polysaccharides or certain polycarboxyl acids are stated to have increased affinity for the lymph system (16). The products have been designated antibiolymphins. In comparison with the corresponding streptomycin and neomycin sulfates, streptolymphin and neolymphin produce lower but more sustained blood levels and much higher and more prolonged lymph levels. Acute toxicity (mouse) is said to be less for "lymphins" than for the corresponding sulfates. The products have been prepared and tested in Czechoslovakia and are not available commercially in the United States.

Antibiotic Prophylaxis.—Apart from the now well-substantiated prophylactic use of benzathine penicillin in cases of known or suspected past history of rheumatic fever, there are few, if any, well-documented indications for antibiotic prophylaxis. Indeed, there is a mounting body of evidence that useless prophylaxis may not only contribute to gradual diminution of the therapeutic efficacy of antibiotics by sensitizing the patient and engendering emergence of resistant strains of bacteria, but may actually delay recovery (17, 18), predispose the patient to virus infections (17, 19), and encourage implantation in the respiratory and urinary tracts of organisms ordinarily uncommon in those regions except as transients or parasites (20).

The high incidence of respiratory disease and resultant loss of time from work or other regular activities has been a major factor in the (unfortunately) common practice of using antibiotics (mainly penicillin and tetracyclines) prophylactically in treating upper respiratory infections. The earliest study of mass prophylaxis (with penicillin) involving 1,486 subjects in the experimental group and 1,451 control subjects for a total of 9,264 patient-months disclosed no significant difference in incidence of respiratory or of nonrespiratory illness in the two groups (21). The argument that antibiotics are given in treating the common cold in pediatric patients to prevent secondary complications has been completely demolished with the demonstration in a series of 217 children that the incidence of secondary complications is the same (about 15%) whether or not antibiotics are given, that complications in untreated patients generally are of bacterial etiology and can be treated effectively with antibiotics if and when they arise while complications in subjects treated prophylactically often are viral and are not amenable to antibiotics, and that the average recovery time for uncom-

plicated cases was 5.8 days for the controls, 7.2 days for the penicillin group, and 8 days for patients treated with one of the tetracyclines (17). Specific treatment of complications at their inception is preferable to prophylaxis at the beginning of fever. Pure cultures of colon bacilli have been found in the throats of children given penicillin prophylactically for the common cold (22). Instances of routine antibiotic prophylaxis appearing to increase rather than decrease infection are numerous (17–26). A sharp increase in death rate (from 29 per 1,000 in 1954 to 144 per 1,000 in 1958) among infants born after premature rupture of fetal membranes has been correlated with prophylactic use of antibiotics (27). The subject of prophylaxis in its various ramifications, including indications and contraindications in various aspects of surgery (26, 28–31) where it was concluded in a survey of many top-ranking hospitals by the Medical Audit Program (32) that in a series of 1,536 patients there was a 74% needless misuse of antibiotics in inguinal herniorrhaphies, has been reviewed (ref. 7, p. 13).

Mixed Therapy.—*Combinations of Antibiotics.*—There are very few clearly substantiated indications for mixed therapy, despite the prevalence of this form of treatment. Outstanding examples that have been well established empirically are concurrent administration of penicillin and streptomycin in some cases of subacute bacterial endocarditis, and joint use of streptomycin and PAS and isoniazid in tuberculosis. In brucellosis, streptomycin or tetracycline given with an appropriate sulfonamide usually produces better clinical results than any one of the agents alone. But apart from these few instances, it is extremely difficult to find objectively critical reports to support administration of many of the antibiotic combinations that have been proposed for general use in other infections. Jones and Finland (33–40) in neither their own laboratory and clinical observations nor in thorough review of the literature could find experimentally demonstrable evidence to support mixed therapy as a general practice. As pointed out in the "New Drugs Development" volume of the 1960 edition of the U.S.D., "their conclusions lend support to the editorial view (41) that 'most often the only justification for including one or another of the components is the fact that it is being produced by a particular manufacturer or that pressure is brought to bear on the management by the sales force to provide some combination to meet effective competition.' "

Except in the three disease entities mentioned above, administration, in adequate dosage, of the more active member of any combination usually produces therapeutic results equal or superior to those produced by the combination. Carefully controlled studies have failed to confirm the subjective claim, vigorously promoted in some advertising, that simultaneous use of two or more antibiotics suppresses emergence of resistant strains of organisms from initially sensitive ones. While it is possible that in some instances emergence of such strains may be slightly delayed, careful studies have shown that it is not completely suppressed and that after repeated exposure to the mixtures, organisms become resistant to both components just as if they had been exposed to the single entities (36, 38, 42). For a possible exception to the general rule about joint use of two antibiotics, see the discussion of novobiocin (below).

Antibiotic-Corticosteroid Mixtures.—Simultaneous use of antibiotics and of corticosteroids, whether for treatment of a single disease entity or of different involvements, is a highly controversial matter. Only a few years ago, a general dictum was that when antibiotics are being employed, corticosteroid hormones are contraindicated. However, successful use of cortisone, to provide subjective improvement, during chloramphenicol treatment of typhoid and typhus fevers was demonstrated in 1954, although the numerous pitfalls, including the false sense of security that the dramatic subjective improvement of the patient may give the physician, were pointed out (43). In general, systemic use of antibiotic-steroid combinations or of the separate entities simultaneously is seldom warranted, and it should usually be looked upon with disfavor. A critical appraisal of the rationale and physiologic complications and of the rare indications and numerous contraindications for such use has been published (44).

While recognizing the inherent danger of systemic antibiotic-corticoid therapy, a number of investigators have recommended such mixtures for local use. Representative proposals are use of antibiotic-steroid mixtures in nebulized sprays for treatment of laryngeal conditions (45), and in ointments for cutaneous infections (46, 47), especially when pyogenic (48). Although the corticosteroids may alleviate irritation and inflammation and may thereby produce a subjective feeling of improvement on the part of the patient and, it is sometimes contended, accelerate healing processes, their use in any infection is potentially hazardous. The inflammatory process is an important first line of defense to localize infection. If this natural process is eliminated or

minimized before the antibiotic takes effect, the possibility of rapid spread of the infection, which may become systemic, is increased. Moreover, there is the possibility that some of the steroid may be absorbed and induce systemic effects of its own, and care must be exercised in selection of the appropriate compound. It has been reported that hydrocortisone decreases resistance to infection and depresses immune responses, while corticosterone, in the same dosage, does not (49).

Antibiotics and Gamma Globulin.—In another category of mixed therapy, joint use of gamma globulin and antibiotics, particularly chloramphenicol, has been reported to produce better response than antibiotics alone (50, 51).

The pros and cons of mixed antibiotic therapy have been reviewed and editorialized extensively (52–59).

Antibiotics as Growth Stimulants.—Certain antibiotics were shown as early as 1946 to have a pronounced growth-promoting effect on the young of some animals (60), and today fortification of feeds, especially for poultry and swine, is routine in animal husbandry and poultry raising. The subject of antibiotics in animal nutrition has been reviewed thoroughly (61).

Following demonstration of the beneficial effect of antibiotic-supplemented feeds on growth of young animals, it was natural that similar studies should be conducted with humans. Chlortetracycline, used in small amounts as a diet-supplement, has been reported to stimulate growth of grossly undernourished children (62), and tetracycline used similarly has been reported to produce marked improvement in growth of dystrophic infants (63).

ANTIFUNGAL ANTIBIOTICS

Amphotericin B

Trade Name: Fungizone (Squibb).
Biologic Origin: Streptomyces sp. (M-4575 in Squibb culture collection) isolated from soil collected in Orinoco River region of Venezuela.
First Reported: 1956 (64).
Clinical Use: antifungal in systemic and superficial mycoses; also to reduce or prevent yeast or monilial overgrowth during tetracycline therapy.
Usual Dose; Intravenous Infusion: 1 mg./Kg./day, the daily dose being dissolved in 1 L. of 5% glucose and infused over a minimum time of 6 hours. This route is more reliable than the oral route. *Oral:* 2 Gm./day for adults has been effective for coccidioidomycosis and other disseminated fungus diseases. Doses up to 5 Gm./day have been used. Daily dose is divided when drug is given orally.
Toxicity: Appears to be low, but nausea, diarrhea, anorexia, and mild reversible azotemia have been reported.

Chemistry.—Amphotericin consists of two closely related compounds, amphotericins A and B. The complete molecular structures are unknown, but the empirical formula, $C_{46}H_{73}NO_{20}$, has been determined for the B fraction. The chemical literature has been reviewed (65, 66).

Amphotericin B, which is several times more active than the A fraction against fungi *in vivo* (67) and is the form that is regularly available, is a lactone-containing polyene structure (68), a type of configuration that has particular interest because of its presence in a number of antibiotics that exhibit antimycotic activity. The aminodesoxyhexose, mycosamine, which occurs among the degradation products of nystatin, is also an important moiety of amphotericin B. Chemical evidence suggests varying degrees of relationship between amphotericin and nystatin and some of the less well-known antifungal antibiotics, e.g., candicidin, filipin, fungichromin, pimaricin, rimocidin, and trichomycin.

The amphotericins are soluble in lower alcohols and in water-saturated butanol, but are less soluble in anhydrous butanol and in water (69). Both amphotericins are stable at moderate temperatures when dry and protected from light and air. In solution, stability is closely related to temperature and pH, maximum stability being in the neutral range.

The biologic activity of amphotericins is markedly affected by pH. The MIC of amphotericin B against several test organisms decreases from about 2.5 mcg./ml. at pH 4.5 to about 0.02 mcg./ml. at pH 8.0 (70), but there is a plateau of more or less uniform activity between pH 6.0 and 7.5. Therefore, assays are generally performed in media adjusted to a value within that range. Various methods for assaying amphotericins biologically, using *Saccharomyces cerevisiae* and *Candida albicans* as test organisms, have been developed (64, 70–72), including techniques for specifically determining amphotericin B activity in serum (70) and other body fluids (72) and in mixtures of the A and B fractions (72).

Absorption and Excretion.—Although amphotericin is poorly absorbed from the gastrointestinal tract, concentrations several times those required to inhibit several species of pathogenic fungi *in vitro* can be achieved in the blood following oral administration (71). The drug disappears from the blood slowly, and concentrations from 30 to 80% of the maximum achieved may still be present 12 or more hours after the last dose. However, continued dosage does not lead to significantly increased concentration. Once a daily dose of 2 Gm. has been reached, very little

increase in blood level is achieved by increasing the daily intake.

Higher blood levels are produced by intravenous infusion than by oral administration, and even 20 hours after infusion blood levels above the maximum achieved by oral administration may be expected. Significant amounts of the drug are absorbed from the plasma by the red cells (73) and small amounts appear in the spinal fluid after infusion of 0.37 to 1.0 Gm. daily.

Absorption of intramuscular doses is insignificant (70).

Total 24-hour urinary output of amphotericin B usually ranges from about 100 to 300 mcg. when patients are given a 5-Gm. oral dose daily; slightly larger amounts being eliminated if a mixture of the A and B components is given. On an intravenous infusion regimen, about 5% of the total daily dose appears in the total 24-hour urine. However, excretion continues for several days after the last dose is administered, and as much as 40% of the total dose may ultimately be recovered. Blood levels up to 20% of the maximum achieved during therapy may still be present a week after treatment is discontinued.

Clinical Uses.—The most significant use of amphotericin is in treatment of the systemic mycoses, prognosis of which, until the advent of this drug, was usually poor and always entailed, at best, a long period of illness and of convalescence. Among such infections successfully treated with amphotericin are cryptococcosis (74–76), histoplasmosis (76, 78, 79), blastomycosis (76, 77), coccidioidomycosis (76, 80–84), *Candida albicans* peritonitis (85), and American leishmaniasis (86). See also the proceedings of the New York Academy of Science's "Second Conference on Medical Mycology" (86a).

The duration of infection before amphotericin was started has ranged in a number of cases from 1 month to 10 years (74, 80). Among six cases of cryptococcal meningitis clinically rated "poor," "critical," "paraplegic," or "moribund" at the start of treatment with 50 to 100 mg., intravenously infused in 1 L. of 5% glucose over a period of 6 hours daily for total doses of 1.4 to 3.7 Gm., 5 survived (74) and, of these, 3 were considered "normal" on the basis of clinical criteria and spinal fluid findings, 1 was considered improved, and the paraplegic case was rated "improved" in terms of spinal fluid findings, although "unchanged" in clinical status. Other investigators have administered oral doses (4 to 5 Gm./day) in treating cryptococcosis, histoplasmosis, blastomycosis, and coccidioidomycosis. A number of patients who failed to respond satisfactorily to oral therapy have been rated "apparent recovery" or "objectively improved" after intravenous therapy, and, in general the intravenous route is preferred because of its greater reliability.

In all cases, treatment is a prolonged affair, ranging in the instances cited from about 1 to 14 months. Unequivocal evaluation of antimycotic agents for systemic infections is a slow process because of the long follow-up periods necessary to establish cure indisputably rather than mere arrest. Results of studies of experimental infections in animals have suggested that in nontoxic doses amphotericin is fungistatic rather than fungicidal (87, 88). However, there is much interspecies variation in sensitivity to drugs and, pending more information, it cannot justifiably be assumed that the same conclusion applies to treatment of human infections.

Amphotericin B, formulated in an ointment, has been considered curative in infants with cutaneous candidiasis in the diaper area and in guinea pigs experimentally infected with *Trichophyton mentagrophytes* (67).

Given orally, the antiobiotic has been reported effective in checking and depressing tetracycline-induced overgrowth by *Candida albicans* in the gastrointestinal tract (89–94). A tetracycline-amphotericin ratio of 5:1 has been recommended (92). Mysteclin F (Squibb) is a preparation incorporating the two antibiotics in a single preparation in this ratio. Despite the reduction induced in the fecal load of *Candida*, amphotericin seems to have little or no effect on the number of *Candida* organisms recoverable from throat swabs and sputum (90).

Unessential use of any antibiotic is undesirable for many reasons, several of which were mentioned above in the discussion of prophylaxis and of mixed therapy. Therefore, since not all patients receiving tetracycline develop candidial or monilial overgrowth, prophylactic use of amphotericin during tetracycline thrapy should be limited to classes of patients experience has shown to be particularly prone to such side effects, i.e., the elderly, the debilitated and/or undernourished, the pregnant, and the menstruating.

A mixture of amphotericin B (50 mg.) and neomycin (1 Gm.), given on an appropriate schedule, has been rated "one of the better drugs for preoperative preparation of the colon" in elective surgery (95).

Amphotericin may become an important adjunct in tissue culture for virus propagation.

At a concentration of 20 mcg./ml., it suppresses growth of species of *Candida, Rhodotorula, and Aspergillus* as effectively as 100 mcg. of nystatin (96).

Resistance.—No evidence of increased resistance of initially sensitive fungi ascribable to exposure to amphotericin in clinical practice has been reported. However, varying degrees of resistance can be induced *in vivo*, depending on the species and strain of fungus studied, the culture medium employed, the frequency of transfer, and other experimental variables (97–99). *In vitro*, there is cross resistance between amphotericin and nystatin (97). This is not surprising in view of the chemical relationship of the two drugs.

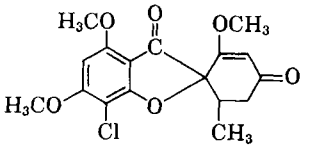
Toxicity.—Incidence of untoward side effects, either acute or residual, during clinical use of oral amphotericin appears to be low. However, experience with the drug is still relatively limited, in comparison with older antibacterial antibiotics. A dose of 2 Gm. daily given orally for 14 months has been used "with no subjective or objective side effects" and "no evidence of hepatic, renal, or hematopoietic reactions" (100). However, in a few instances "mild and reversible" azotemia, nausea, diarrhea, anorexia (76), and "transitory impairment of renal function, ... suggestive of nephrotoxicity" (101) have been reported. In general, the early impression of low clinical toxicity has been supported by more recent studies (86a) and is consistent with the results of pharmacologic and toxicologic studies on animals (102). Incidence of untoward effects may be considerably higher via the intravenous route.

Griseofulvin

Trade Name: Fulvicin (Schering), Grifulvin (McNeil). Also available as Griseofulvin (Ayerst).
Biologic Origin: Penicillium griseofulvin, P. janczewskii, P. nigricans.
First Reported: 1939 (98).
First Clinical Use: 1958 (116).
Clinical Use: Systemic treatment of superficial dermatomycoses. Nocardiosis, sporotrichosis, chromoblastomycosis, and deep infections with *Actinomyces bovis* have been reported to respond occasionally to griseofulvin, but generally it is not considered effective in systemic or deep mycoses.
Usual Dose; Adult: 250 mg. 4 times daily by mouth. In severe infections, 2 Gm. daily, in four divided doses, until clinical response is evident, then reduced to 1 Gm./day. *Children:* If over 50 lb., 250 mg. 3 times daily. *Infants* (Under 2 years): 125 mg. twice daily or, alternatively, 10 mg./lb. of body weight/day.
Toxicity: Apparently low, but gastrointestinal upset, diarrhea, headache, and occasional confusion, exhiliration, and insomnia have been reported. Decreased systolic blood pressure and lymphocytosis also have been reported. Side effects generally have not been severe enough to warrant discontinuance of the drug.

Historical Comment.—Griseofulvin, an antifungal compound originally discovered in 1939 among the metabolic products of *Penicillium griseofulvin* (103) and subsequently of other species of *Penicillium* (104, 105) was studied intensively by phytopathologists for nearly 20 years, as a potentially useful agent for systemic control of fungi pathogenic on plants, before it was investigated for possible usefulness in treatment of mycotic infections of animals. The antibiotic, also known as the "curling factor" because of the characteristic morphologic aberrations it causes in the germ tubes of sensitive fungi (106), is stated to affect only those species that have chitinous cell walls and to be ineffective against species that have cell walls of cellulose or of other nonchitinous material (107), but this view has been questioned. The uses of griseofulvin in plant pathology have been reviewed (108).

Chemistry.—The empirical formula, $C_{17}H_{17}ClO_6$, originally proposed (103) has been confirmed (109, 110), and the following structure has been developed (111).



Griseofulvin

Griseofulvin is only slightly soluble in water (0.001% at pH 7.0). It is soluble to the extent of 0.1% in methanol and ethanol (107). It is thermostable over the pH range 3.0 to 8.8 at 25° for at least 4 weeks and at pH 7.0 withstands autoclaving at 250° F. for 30 minutes. When dry, it is stable at 100° F. for at least 20 months (112).

Absorption and Excretion.—Orally administered griseofulvin is absorbed rapidly and excreted slowly. In experiments with dogs and guinea pigs, little or no antibiotic was found in urine collected within 24 hours after first administration of doses ranging from 0.1 to 2.0 Gm./Kg./day, and there was no significant cumulative excretion (113). During the third week of a continuous feeding experiment, there was little difference in total daily excretion of drug whether animals were dosed at 0.1, 0.2, or 0.5 Gm./Kg.

In another experiment, excretion continued for 1 week after the last of 3 days of dosing with 1 Gm./Kg. About 0.5% of a single oral dose of 50 mg./Kg. given to guinea pigs was recovered from feces collected during the first 24 hours, but

none was found in feces excreted during the next 24 hours. The doses used for these studies were well above those (15 mg./Kg./day for 5 to 15 days) that were curative for *Trichophyton mentagrophytes* infections in guinea pigs.

Data similarly indicating that only a small fraction of griseofulvin ingested by human beings is excreted in the urine have been reported (114), although others have reported recovery from urine of 50% or more of an orally administered dose (115).

Clinical Use.—Clinical interest in griseofulvin and its use in medicine stemmed from the demonstration in 1958, nearly 20 years after discovery of the drug, that, administered orally in adequate dosage, it provided effective and safe systemic protection for guinea pigs experimentally infected with ringworm (*Microsporum canis*) or with *Trichophyton mentagrophytes* and that it was curative even when the onset of treatment was delayed until the infection was well established (116). After a few days of treatment of ringworm, the fungus can be found only in the upper portions of the hair shaft; the lower portion, formed after initiation of treatment, grows out free of infection, presumably because of the presence of griseofulvin in the hair. That the antibiotic is actually present in hair after oral administration can be demonstrated by extraction with hot methanol (117). The drug proved to be effective in curing spontaneous *Trichophyton verrucosum* infections in calves (118), *Microsporum canis* infections in cats (119), and *M. canis* and *T. mentagrophytes* infections in dogs (120), and this led to the first trials in human medication.

The efficacy of griseofulvin as a systemic antidermatophyte in humans has been well established (114, 115, 120 136a). The following dermatophytes that commonly infect hair, nails, and skin are susceptible to it: *Trichophyton interdigitale*, *T. mentagrophytes*, *T. rubrum*, *T. schoenleini*, *T. sulfureum*, *T. tonsurans*, *Microsporum audouini*, *M. canis*, *M. gypseum*, and *Epidermophyton floccosum* (112), and it has been stated that the antibiotic " is active in low concentration against all the common dermatophytes" (116).

Infections caused by any of the above fungi in adults generally yield to an oral dosage of 250 mg. every 6 hours. In children, the total daily dose (but not the number of doses per day) may be reduced. (See capsule summary above.) Improvement and incipient clearing may occur soon after initiation of treatment, but cure may require several weeks or even months of continued dosage.

The rapidity of response to griseofulvin depends on the site and duration of infection. Tinea capitis may be "clinically cured" in 3 to 7 weeks, but if there is follicular involvement response generally is slower, and 3 to 4 months of daily dosage may be required (129). Infections of the glabrous skin may be cleared in 2 to 4 weeks, infections of the soles and palms in 2 to 8 weeks, when fingernails are involved 10 or more weeks may be needed to effect "clinical cure," and when toenails are infected an even longer time (123). Evidence of improvement in onychomycosis usually is seen within 2 weeks in fingernails and in 3 to 4 weeks in toenails, although complete cure may require several months, due to the relatively slow growth of nails, estimated at about 1 mm./wk. for fingernails and about 0.5 mm./wk. for toenails (112).

Topical application of griseofulvin may effect some improvement in dermatophytic infections, but generally the results are not as satisfactory as when the drug is given orally (137–139). This may be because griseofulvin probably does not eradicate the dermatophyte from infected hair, skin, or nails but rather, by its presence in the new tissue as it forms, renders the new tissue resistant to invasion. When the drug is applied topically, little, if any, occurs in the tissues. However, one group reported erratic response of tinea pedis to oral griseofulvin but good response to topical application (140). This is contrary to the majority of reports.

Although markedly effective against the superficial mycoses affecting skin, hair, and nails, griseofulvin generally is ineffective in deep mycoses in humans, when given orally in doses of 0.5 to 2.0 Gm. daily (138), a conclusion which is supported by the results found in experimental blastomycosis, histoplasmosis, cryptococcosis, and coccidioidomycosis (141). However, others have claimed "some therapeutic effect" in nocardiosis and sporotrichosis (142, 142a), in chromoblastomycosis (143), and perhaps in actinomycosis (128).

Resistance.—There are natural variations in susceptibility of fungus species to griseofulvin, even among the genera of dermatophytes that are most sensitive. However, although resistance can be induced *in vitro*, there is no evidence to date of emergence of strains with increased resistance from initially sensitive strains as a result of clinical use of the antibiotic. Occasionally, *C. albicans* has seemed to become dominant and to cause intertriginous dermatitis of groins and/or toe webs as the original griseofulvin-sensitive dermatophyte has been inhibited (129).

Toxicity.—Experiments on animals and clinical observation on humans indicate that toxicity of griseofulvin is low, both in incidence and severity. Mice survive oral doses of 50 mg./Kg. (112), but massive intraperitoneal doses (2 Gm./Kg.) cause severe damage to the seminal epithelium in rats, and intravenous doses of 100 to 200 mg./Kg. (about 10 times the therapeutic dose) cause arrest of mitosis at metaphase (144).

The comment "the incidence of toxicity and side reactions (with therapeutic doses in humans) is extremely low" (130) is typical of the majority of clinical reports. In most patients on whom laboratory studies have been performed, there has been no change in urinalysis, hemoglobin levels, differential counts, hepatic function, and no constant trend in variability of sperm counts. Occasionally, a transient decrease in total leukocyte count has been noted but frequently this has returned to normal despite continued medication.

However, in some instances, about 30% according to one author (145), a relative lymphocytosis has appeared and in some there has been mild albuminuria, although kidney function tests revealed no abnormality. Other side effects occasionally reported have been elevation of icteric index without visible evidence of jaundice, diarrhea, fatigue, vague feelings of gastrointestinal uneasiness after the first 3 to 7 days of treatment, a feeling of "fullness" in the head, confusion, exhiliration, and insomia. Incidence of these effects has been less than 2% and generally they have disappeared even though medication was continued. It has been suggested that some of the effects may have been psychogenic. Imbibing of alcohol while under griseofulvin treatment has been reported to lead to "tachycardia and a flush" (112), and morbilliform eruptions with temperature elevation have been noted (145).

An effect which probably is not of psychogenic origin is the reported 10 to 15% decrease in systolic blood pressure without any subjective symptoms. In the only report on this effect, it was observed in 26 of 52 patients studied (112).

There is no question of the tremendous value of griseofulvin in treating dermatomycoses. It fills a long-standing desire of dermatologists for a relatively safe drug for systemic treatment of such infections from within outwards to replace, or at least complement, the older and sometimes disappointing attack from the outside via topical medication. However, as the drug becomes more widely used, side effects and details not yet recognized may be revealed. The reported decrease in systolic pressure would be an interesting facet to pursue.

Nicetin

Nicetin is the Parke, Davis & Company trade name for the antifungal substance formerly designated CMA-37. This intensely bitter, light yellowish crystalline compound, which is soluble in water or in physiologic saline only to the extent of 0.125%, is closely related to chloramphenicol in chemical structure, but differs markedly from it in antibiotic activity.

Chemically, Nicetin differs from chloramphenicol only in having a ketone group substituted for the secondary alcohol group of the latter. But, in contrast to chloramphenicol, which is a potent antibacterial and antirickettsial drug with no significant action against fungi, Nicetin is effective in moniliasis and in some infections of skin and nails, but has little activity against bacteria.

In the first clinical trial, a case of bronchopulmonary moniliasis which had failed to respond to all other treatments and in which the patient was failing rapidly was given 1 Gm. of Nicetin on the first day and then 250 to 500 mg. 3 times daily. Favorable response occurred almost at once, and after 6 weeks of treatment the patient was free of cough and other symptoms and had gained weight (146). A 2% ointment of Nicetin and a 2% solution of the drug in propylene glycol were found far superior to ammoniated mercury and other common agents in treating 75 cases of various fungus infections of the skin, scalp, and nails (147). Desirable features of the drug are that it is fungicidal, not merely fungistatic, and that it is keratolytic, which facilitates its penetration in the deeper layers of infected skin.

Since Nicetin is so closely related chemically to chloramphenicol which has been alleged although not proved to induce aplastic anemia and other blood dyscrasias, systemic use, except in extreme situations unresponsive to other treatment, would be ill advised. Urinary frequency, dysuria, and hematuria have been observed when doses of 250 to 500 mg. of Nicetin have been given thrice daily. These effects have been ascribed to a chemically-induced hemorrhagic cystitis (147).

Two of 75 patients treated topically with 2% Nicetin (in ointment or in solution in propylene glycol) developed allergic reactions consisting of "intense edema" and marked erythema of the treated limb within 4 to 8 hours (147). The symptoms were controlled with antihistamines or with prednisone. It was not determined whether the reaction was a direct allergic response

to the drug or resulted from the sudden release of large amounts of allergenic lysis products from the invading fungus. Nonetheless, it was recommended that topical treatment not be continued for more than 10 days.

Note: Nicetin is not currently available commercially.

ANTIBACTERIAL ANTIBIOTICS

It is not difficult to find microorganisms that produce compounds antagonistic to bacteria. Despite the literally hundreds of antibacterial antibiotics that have been isolated and described, it still is not too difficult to discover a new one. But to find one that is clinically useful enough to warrant industrial production is becoming extremely difficult. To warrant production in the face of competition from the established agents, such as penicillin, the tetracyclines, chloramphenicol, etc., a new antibiotic must have some special characteristic that the others lack. One such characteristic is the ability to inhibit organisms, especially so-called "hospital" staphylococci, that have become resistant to the older antibiotics that have been prescribed with such abandon for a decade or more.

Kanamycin, novobiocin, ristocetin, and vancomycin all came into production during the period covered in this survey and were developed for marketing primarily because of their activity against penicillin- and tetracycline-resistant staphylococci. Frequently these antibiotics are no more active than the older ones against the common strains of *Staphylococcus*, and in some instances they are less active. It is to be hoped that restraint and responsibility will be exercised in prescribing these newer drugs, limiting their use to situations individually demonstrated to be unresponsive to the older agents, so that their effectiveness can be preserved. Many hospitals, through their own self-policing, have restricted use of novobiocin, ristocetin, vancomycin, and others to such situations.

Colistin attracts attention primarily because of its activity against recalcitrant Gram-negative bacteria and paromomycin, discovered in 1959, has been developed to the production stage primarily because of its special value in amebiasis, shigellosis, and salmonellosis.

Finally, several modifications of other antibiotics, e.g., semisynthetic penicillins and various derivatives of tetracyclines, have been developed during the past five years.

Colistin

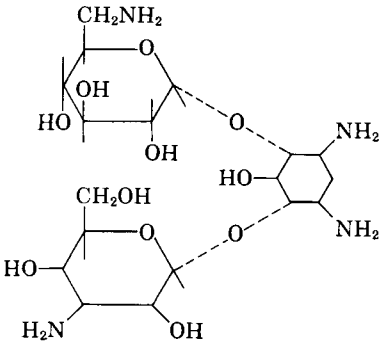
Trade Name: Coly-Mycin (Warner-Chilcott).
Biologic Source: *Bacillus (Aerobacillus) colistinus*, isolated from soil collected in Fukuchima prefecture, Japan.
First Reported: 1950 (147a).
Clinical Use: Urinary tract infections due to Gram-negative organisms, including especially species of *Pseudomonas* but, in general, excluding species of *Proteus*. Also useful in respiratory, surgical, wound, burn, or other infections caused by sensitive organisms.
Usual Dose: 2.5 mg./Kg./day (1.1 mg./lb./day) in 2 to 4 divided intramuscular doses. In severe infections, a large total daily dose may be needed but the total daily dose should not exceed 5 mg./Kg. (2.3 mg./lb.).
Note: Orally administered colistin is poorly absorbed. Therefore, for systemic treatment, intramuscular injection is indicated.
Toxicity: No major toxic effects have been reported in patients receiving normal therapeutic doses. Occasional mild nausea, dermatitis, drug fever, and circumoral paresthesia have been noted but have subsided spontaneously upon withdrawal or reduction of the dosage. Mild pain at the injection site also has been reported.
Comment: Colistin, originally discovered in Japan in 1950, had been extensively studied and used clinically in Japan, Italy, and France before being introduced in the United States in 1959. Extensive references in the Japanese and European clinical literature are cited (147b). Colistin is similar to (and may be identical with) polymyxin E (147c, 147d).

Kanamycin

Trade Name: Kantrex (Bristol).
Biologic Origin: *Streptomyces kanamyceticus*, isolated from soil collected in Nagano prefecture, Japan.
First Reported: 1957 (148, 149).
Clinical Uses: Staphylococcal infections resistant to commonly used antibiotics. Refractory urinary tract infections, especially those due to staphylococci, *E. coli*, or gonococci. May be beneficial in *Proteus* infections but not indicated when *Pseudomonas* or enterococci are the etiologic agents. Tuberculosis may respond in favorable cases, but the drug is not impressive in advanced cases, especially those that have failed to respond to other drugs. *p*-Aminosalicylic acid should be used jointly with kanamycin.
Usual Dose: In acute or chronic infections, 1 to 2 Gm. daily, injected in 2 to 4 equally divided doses, or a dose of 15 mg./Kg./day may be used in divided doses. Total dose in any one course of treatment should, if possible, not exceed 40 Gm. For extended use in chronic illness, dose should not exceed 6 Gm./wk. In tuberculosis, a schedule of 1 Gm. twice a day, 2 days a week with PAS, 10 Gm. daily, has been recommended.
Note: For systemic medication, kanamycin must be given by injection, since it is not absorbed well from the gastrointestinal tract. Pain or irritation at the injection site is fairly frequent.
Toxicity: Acute toxicity is low in short-term therapy. Nephrotoxicity is the major untoward effect. Symptoms usually are mild and subside spontaneously when the therapy is terminated. However, upon continued use, as in chronic illness, eighth cranial nerve damage, involving auditory or vestibular function or both may occur.

Comment: Kanamycin was discovered in the laboratories of the Japanese National Institute of Health (Tokyo) in 1957 and was marketed in the United States in 1958. Results of some of the microbiologic and chemical research that preceded release of the drug have been recorded in a single symposium publication (150).

Chemistry.—Kanamycin, related chemically to neomycin and streptomycin, shares some of their antibacterial and pharmacologic characteristics. The molecule contains 2 amino sugars linked glycosidally to 2-desoxystreptamine. The latter is also a moiety of neamine (Neomycin A)



Kanamycin

The chemical research leading to characterization of the compound and elucidation of the above structure has been reviewed (66). A second antibiotic, kanamycin B, occurs in crude preparations (151–153) but is present only in minor amounts, if at all, in medicinal kanamycin.

Kanamycin is stable and water-soluble. It is resistant to acid and alkaline hydrolysis and is thermostable in the pH range of 2.0 to 11.0. At pH 6.0 to 8.0, it withstands boiling for 30 minutes. Detailed data on stability, solubility, and other pharmaceutically important properties have been published (154).

Absorption and Excretion.—Kanamycin is poorly absorbed following oral administration, and this route is not suitable for systemic medication. The drug is well absorbed following intramuscular or subcutaneous (155) or intraperitoneal injection (156).

In humans, kanamycin appears in the blood about 15 minutes after intramuscular injection, and peak levels are attained in about 1 hour on either a single- or multiple-dose schedule. The concentrations fall rapidly after the peak is achieved (157), and blood taken 24 hours after a single injection of 1 Gm. or less generally does not contain assayable amounts of the antibiotic.

In infants and children, intramuscular injection of 5 mg./Kg. produces blood levels roughly comparable to those obtained with 250 mg. in adults, but the concentration tends to fall more rapidly (158).

A progressive build-up of concentration is not seen on a multiple-injection schedule, and after successive doses of 0.25, 0.5, or 1.0 Gm. the blood levels are seldom more than a fraction of the levels achieved 1 hour after the initial injection.

Primary excretion of kanamycin is renal, although some also appears in the bile. The renal elimination appears to be simple glomerular filtration (155, 159). Total 24-hour urinary recovery from human subjects with normal kidney function has been reported as 40% of the total dose for bed patients (160), ranging up to 100% for healthy volunteers (161). These differences may not be as great as appears on the surface (157).

Normally, in patients with good kidney function, there is no build-up of concentration. But on a multiple-dosage schedule, the plasma level may increase abruptly when there is renal impairment, and with the elevated levels toxic manifestations, especially ototoxicity, may be anticipated. Since the drug is also nephrotoxic, it should be used only with extreme caution in subjects with functional renal problems (162). The serum half-life of kanamycin in normal subjects has been estimated to be about 4 hours, but in uremic patients the drug was detectable in the blood for 4 to 5 days after a single dose of 1 Gm. (162). Severe hepatic disease may also cause accumulation of kanamycin, even when the drug is administered orally, despite poor absorption from the gastrointestinal tract (162, 163).

Clinical Uses.—The *in vitro* antibiotic spectrum of kanamycin embraces several Gram-negative and some Gram-positive species of bacteria and various acid-fast organisms, including *Mycobacterium tuberculosis* H 37 Rv. In general, the clinically useful spectrum is similar, provided the drug is administered in adequate dosage for an adequate length of time.

Staphylococcal Infections.—Kanamycin in divided intramuscular doses of 10 to 100 mg./Kg./day was rated "good" in treating mild and moderately severe cases and as "effective" in about 50% of children severely ill with hospital-acquired straphylococcal infections during the 1958 epidemic outbreak in a maternity service in Houston, Texas (165). Most of the strains were resistant to penicillin, streptomycin, and the tetracyclines, but all were inhibited *in vitro* by less than 6.25 mcg. kanamycin/ml. The infections had existed for from 6 hours up to 40 days before initiation of kanamycin, and about two-thirds of the patients had already failed to respond to at

least one, and most of them to two or three, other antibiotics. Other studies, both *in vitro* and clinical, have confirmed the sensitivity of the majority of clinically isolated staphylococci to kanamycin (166–168).

From purely microbiologic considerations, such as sensitivity of organisms, static *vs.* cidal concentrations of drug, etc., neomycin is a more effective antistaphylococcal agent than kanamycin (166, 169), but kanamycin probably is not as toxic as neomycin and, therefore, is preferable to it for systemic medication (170, 171). Kanamycin, ristocetin, and vancomycin can be considered as essentially in the same category for treatment of staphylococcal infections recalcitrant to safer antibiotics.

Urinary Tract.—The rapid appearance of kanamycin, and the relatively high concentrations it reaches, in the urine make it a useful drug for treating susceptible infections of the urinary tract. Good results generally can be achieved when infections are due to *Staphylococcus, Escherichia coli,* or *Gonococcus* (172). *Proteus* infections respond moderately (172) and infections due to *Aerobacter* or to *Klebsiella* can be treated satisfactorily, if the dosage is adequate (173). Others have confirmed these conclusions (174–176). Tuberculous infections of the urinary tract also may respond, if sufficient dosage is provided for an adequate time and PAS is used jointly with kanamycin (172).

Pulmonary Tuberculosis.—Reports from Japan have rated kanamycin "almost equivalent to the streptomycin-PAS combination" and "an excellent antituberculosis drug" (177), but investigators in the United States have not been so enthusiastic (178–180) because of the "toxic potentialities for the eighth cranial nerve and the kidneys, and hypersensitivity reactions," and because of the rapid development of resistance by the tubercle bacilli to the drug.

Other Infections.—Reports are conflicting regarding the effectiveness of kanamycin in *Salmonella* and *Shigella* infections (181–184). Certainly it is not as effective as chloramphenicol in typhoid fever, although it may have some effect in reducing the typhoid-carrier state (183). Administered orally in adequate dosage, it is useful as a preoperative intestinal "antiseptic," giving adequate control of streptococci, coliforms, and *Clostridia* (185–187).

Kanamycin has been reported to be effective in reducing the "cerebral manifestations" resulting from absorption of bacterially formed protein metabolites in patients with cirrhosis of the liver (188), but others have found that the beneficial "blood ammonia changes may not be sustained or reproducible with repeated courses" of treatment (189).

In ointments or creams (0.5%) the antibiotic has been effective in the treatment of cutaneous bacterial infections and has not appeared to be irritant or allergenic (190). However, such use probably should be discouraged as long as effective agents not suitable for systemic administration are available.

Resistance.—There is little or no cross resistance between kanamycin and most of the antibiotics commonly used systemically. However, there is almost complete cross resistance between kanamycin and neomycin and, among strains of *Staphylococcus aureus* and of *Enterobacteriaceae,* there may be complete cross resistance among kanamycin, neomycin, and paromomycin (168) and moderate cross resistance to streptomycin (168). Some cross resistance between kanamycin and streptomycin also appears in *Salmonella typhimurium* (191). Upon repeated exposure, other organisms, including *Mycobacterium tuberculosis,* also develop resistance to kanamycin (192–195).

Views are conflicting regarding emergence of resistant strains under clinical conditions (172, 183, 185, 196–198). The consensus seems to be that microorganisms, other than tubercle bacilli, do not readily develop resistance to kanamycin under clinical circumstances as long as the drug is used cautiously. However, highly resistant staphylococci have been isolated from clinical material (199).

Toxicity.—Results of acute and chronic toxicity studies in animals suggest that kanamycin is somewhat less toxic than streptomycin (155, 200).

Opinions differ concerning chronic toxicity upon extended use in humans, although there is agreement that the drug is potentially nephrotoxic and ototoxic. Donomae (201) considered auditory loss through eighth nerve damage "extremely improbable if the dosage is maintained below 6 Gm. per week," and he stated "there is no other side effect of considerable significance." However, others consider the drug "essentially nontoxic" in doses of 1.5 to 2.0 Gm. daily for less than a total amount of 40 Gm., but caution that when the total exceeds 40 Gm. "there is a clear chance" of permanent eighth nerve damage and deafness (161) and that even with lower total doses signs of renal impairment may be expected during or after treatment (202). Others also have commented on the need for vigilance to detect renal and auditory damage before irreparable

impairment occurs (171, 178, 198, 203, 204). It is clear that toxic effects will occur sooner and be more extensive in patients who have impaired renal function before therapy is started (205, 206) and that eighth nerve damage may continue to progress even after treatment with kanamycin has been discontinued (207).

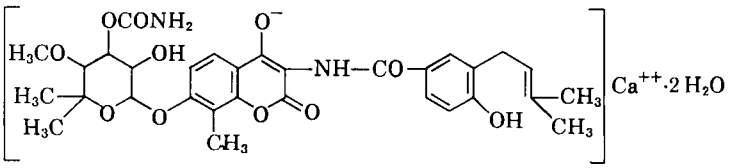
The monopantothenate of kanamycin has been reported less acutely toxic than the sulfate in animals (208), but no studies were made of possible delayed effects.

Irritation, stinging, and pain at the injection site are common (161,198), the incidence possibly being as high as 50 to 60% (198).

Novobiocin

Trade Names: Albamycin (Upjohn); Cardelmycin (Pfizer); Cathomycin (Merck).
Biologic Origin: Streptomyces niveus (*S. spheroides*) isolated from soil collected in Queens Village, N. Y.
First Reported: 1956 (209, 210).
Clinical Use: Staphylococcal infections resistant to the antibiotics in common use. Limited applications in other infections (see text).
Dose; Adult: average 250 mg. orally every 6 hours or 500 mg. every 12 hours; in severe infections, 500 mg. every 6 hours or 1 Gm. every 12 hours. *Children:* 15 mg./Kg. daily in 4 divided doses or, in severe infections, 30 to 45 mg./Kg. daily in 4 divided doses. Prolonged treatment with high doses may lead to skin rashes and other complications.
Note 1: There is considerable variation from patient to patient in serum binding of the drug. Therefore, dosage should be determined on an individual basis.
Note 2: Because of dermatologic side effects induced if the drug is used in too large a dose or for too long a time, the minimal dose capable of coping with the infection should be employed. To be weighed against allergenic potential of the drug is its potential for inducing emergence of resistant strains of bacteria if used in sub-bactericidal amounts. Five hundred milligrams every 12 hours has been recommended as a safe middle course for adults.
Toxicity: Principal manifestations are skin rash, urticaria, and drug fever. Leucopenia also has been reported. Very high doses may induce liver damage. When properly used, the incidence of skin eruptions is relatively low, but precautions are necessary. (See Note above and also text which follows.)

Chemistry.—Novobiocin forms both acid and neutral salts. The calcium salt has the following structure



Calcium Novobiocin

The extensive literature on the chemistry of novobiocin has been reviewed (66).

Absorption and Excretion.—Novobiocin is not readily absorbed via the oral route (211), but the calcium and sodium salts are, as is dihydronovobiocin. The drug is absorbed following intramuscular injection also, but frequently causes appreciable reaction in muscle or subcutaneous tissue when so administered. In the discussion that follows novobiocin refers to the sodium or calcium salt.

Maximum serum levels are achieved within 2 to 4 hours after oral administration. According to Finland and Nichols (212) higher blood levels are achievable with novobiocin than with any other antibiotic given in comparable dosage. However, there is much patient to patient variability, and after a single dose of 250 mg., maximum serum levels may range from less than 3 to about 20 mcg./ml.

On a multiple-dosage schedule, there is some accumulation during the period of the first few doses, but the serum levels soon stabilize and only minor fluctuations occur when 500 mg. of the sodium salt are given at 6 to 8-hour intervals. Peak concentrations are higher and are reached earlier when the drug is given on an empty stomach. Following absoprtion, it quickly appears in almost all body tissues and fluids except the cerebrospinal fluid.

Novobiocin is excreted in the bile, urine, and feces. Major excretion is via the feces. Much of the fecal novobiocin probably arrives there via the bile, following hepatic concentration and elimination, but some probably represents unabsorbed drug. The sustained high blood levels novobiocin reaches may be brought about by reabsorption from the intestine of drug which, having been previously absorbed, has passed through the liver and again into the intestine via the bile.

Urinary excretion accounts for about 3% of the total administered dose (213). Concentrations are usually higher in the urine than in the serum, but the range of concentrations is not so great.

Clinical Uses.—Generally, the monobasic sodium salt is prescribed for adults; the neutral

calcium salt, suspended in syrup, often is preferred in pediatric practice.

Novobiocin is active principally against staphylococci and other Gram-positive organisms, although it has limited activity against some strains of selected Gram-negative species. Certainly, its major usefulness is in treating staphylococcal pyodermas insensitive to other antibiotics (214–218), and some investigators believe use of the drug should be restricted to such situations (215).

Novobiocin has been reported effective also in pneumococcic pneumonia (219), undulant fever (220), and in genitourinary infections due to *Bacillus proteus*, *Micrococcus aureus*, or *Streptococcus faecalis* (221). However, others have not been impressed with its performance in nonvenereal urinary infections by a variety of organisms (222, 223), and it has been declared ineffective in acute gonorrhea (224) and in primary and secondary syphilis (225).

In ophthalmology, novobiocin in a concentration of 1% is not irritating and does not inhibit corneal regeneration (226), and it is useful in treating ocular infections caused by susceptible organisms, especially staphylococci (227, 228).

Resistance.—There is a "definite tendency" for bacterial strains of "increased resistance" to appear, when novobiocin is used alone in clinical practice (229, 232). Laboratory data indicate that a one-step increase of ten-to thirtyfold may occur (230) but that a "synergistic" action and marked retardation of emergence of resistant bacterial strains occur when neomycin or bacitracin (230) or chloramphenicol, chlortetracycline, oxytetracycline, streptomycin, or penicillin (209), and novobiocin are used concomitantly. Synergism between novobiocin and tetracycline and novobiocin and nystatin in treating amebiasis also has been noted (231). These observations have led to the recommendation that another antibiotic to which the etiologic agent is initially sensitive should always be used jointly with novobiocin, especially when a prolonged period of treatment is anticipated, e.g., suppurating lesions or staphylococcal abscesses.

It should be cautioned that in selecting drugs for combined use, attention must be given to a third component, namely, the organism, in the complex system. Two antibiotics which act synergistically against one species or strain of microorganism might be indifferent, or even act antagonistically, against a different species with different metabolic requirements and pathways. Moreover, experience with other mixtures indicates that although emergence of resistant forms may be retarded, it is seldom eliminated and there is, therefore, always the possibility of producing organisms resistant to both drugs instead of just one.

Toxicity.—Novobiocin probably has a low order of toxicity when properly used, although the incidence of untoward complications has been reported to be as high as 12.7% in one study (232) and 18.7% in another (233). High doses were used in both series of patients. The most common side effect is transient skin rash or other dermatologic manifestation which disappears when the drug is withdrawn or therapy is terminated. A more serious effect is leucopenia, the incidence of which is said to be about 1% (234). An elevated icteric index also may arise, although it is not clear whether this is due to bile or to a yellow pigment metabolite of the drug (235). But in any event, the pigment may interfere with determination of serum bilirubin. Thrombocytopenia also has been reported (236).

That these are not constant features of novobiocin therapy is indicated by the report of a patient who received 250 mg. daily for 6 months after a priming dose of 250 mg. four times daily for 3 days and whose icteric index and blood cell count remained normal throughout the treatment (237). Despite a history of allergy to other drugs, the patient showed no evidence of allergy to novobiocin.

Animal experiments have demonstrated severe degenerative changes in liver and kidneys when high doses (300 mg./Kg./day) have been given.

While some authors have reported "remarkable freedom from side effects" (238), others have commented that eruptions were of "such alarming proportions to the patient and . . . physician . . . that the resultant effect was to curtail the number of patients available for study" (239).

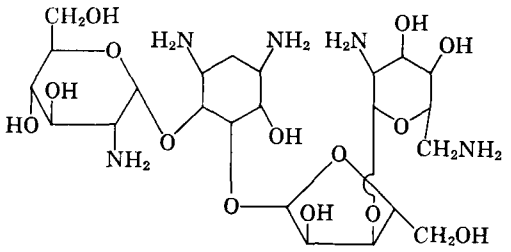
In a comprehensive survey, involving 10 groups of clinicians and aimed at resolving the differences of opinion regarding toxicity of novobiocin, the overall incidence of skin rash was found to be 8.9%, and it was concluded that eruption is likely to occur only when the drug is given in a dose of 2 Gm. or more/day for 6 days or more (240). It was recommended that, in view of the therapeutic effectiveness of the drug in low concentrations against susceptible organisms and the ease with which high blood levels are obtained, 0.5 Gm. of novobiocin given orally twice daily should be an adequate dosage. It was estimated that on such a regimen the incidence of skin rash should be less than 1%.

Paromomycin

Trade Name: Humatin (Parke, Davis).
Biologic Origin: Streptomyces rimosus forma *paro-*

momycinus, isolated from soil collected in Colombia.
First Reported: 1959 (241).
Clinical Uses: Amebiasis, salmonellosis, and shigellosis. Also useful in management of hepatic coma and possibly clearing of typhoid and shigella carriers.
Usual Dose; Amebiasis: Adults 0.75 to 1.0 Gm. (or children 22 mg./Kg.) orally in divided doses for 5 days. Larger doses have been used for both children and adults with no ill effects. *Hepatic Coma:* 4 Gm. or more daily, in divided doses for 5 to 6 days. *Bacillary Dysentery and Nonspecific Diarrhea in Children:* Doses of 50 mg./Kg./day, in divided doses for 5 to 7 days.
Note: Paromomycin is not absorbed significantly from the gastrointestinal tract. Hence, in the conditions cited, the full effect of oral doses is exerted on organisms in that system.
Toxicity: Toxicity is low. In normal subjects and in patients free of diarrhea or dysentery, loose stools generally develop if the daily oral dose exceeds 2 Gm. for more than 3 days. On a dose of 4 to 10 Gm. daily, moderately severe diarrhea occurs, but other untoward effects have not been noted.

Chemistry.—Paromomycin is a stable, amorphous, white substance that is very soluble in water, moderately soluble in methanol, and sparingly soluble in absolute ethanol. It is optically acitve (242). Chemical studies (243, 244) leading to formulation of the following structure have been published.



Paromomycin

Paromomycin is basic and readily forms salts with acids, especially mineral acids. For clinical use, it is prepared as the sulfate.

Absorption and Excretion.—The drug is poorly absorbed following oral administration. In monkeys 1 to 3.5% of an oral dose can be recovered from the 24-hour urine, but up to 17% of an intravenous dose can be recovered in the same period (241). Rapid and efficient absorption follows subcutaneous administration in hamsters, and the drug persists in the plasma for about 4 hours (243). In humans, it is used only via the oral route.

Clinical Uses.—Paromomycin is active *in vitro* against a wide array of Gram-positive, Gram-negative, and acid-fast bacteria, including human type tubercle bacilli (241, 245). It is amebicidal also, either in the presence or absence of bacteria. By use of drug-resistant bacteria in the same culture with amebae, it has been shown that the drug is a direct-acting amebicide (243).

Since paromomycin is not absorbed from the intestinal tract, it is useful via the oral route for treatment of diseases that are caused by susceptible organisms and require vigorous antimicrobial action in the gut. It has been successful in amebiasis (246–252) and in bacillary and nonspecific diarrhea in children. It has proved effective, in a 5-day course of treatment (40 mg./lb.), not only in curing the primary infection in shigellosis and salmonellosis, but also in clearing carriers of the organisms (253). Typhoid carriers, previously treated with chloramphenicol, have been cleared by use of paromomycin (253).

A dose of 500 mg. given 4 times daily for 4 days is effective in the preoperative preparation of the colon for elective surgery.

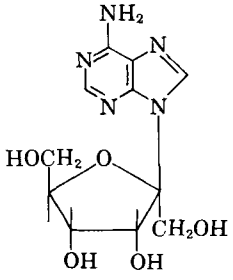
Paromomycin is effective also in management of hepatic coma, unless some liver failure or massive uncontrolled gastrointestinal bleeding is underlying (254), and it may be possible to restore protein safely to the diet sooner when this drug is used than under the conventional treatments (255, 256).

Toxicity.—Toxicity is low. See capsule sketch at the beginning of this section and, for details, reference 241.

Psicofuranine

Psicofuranine, discovered in 1959 in culture filtrates of *Streptomyces hygroscopicus* var. *decoyicus* in the Upjohn Laboratories (257–259), is unusual in three respects: (*a*) it is a nucleoside antibiotic, (*b*) it has both antibacterial and antitumor activity, and (*c*) it has a low order of antibacterial activity *in vitro*, as tested in common laboratory media, but is highly effective in protecting experimentally infected animals. It is not commercially available at present.

The compound is very soluble in dimethylformamide, dimethylsulfoxide, and hot water. At room temperature, water solubility and methanol solubility are about 8 mg./ml. (257). It has been identified as 6-amino-9-D-psicofuranosylpurine (257) having the following structure



Psicofuranine

which is the same as that proposed for angustimycin (260), discovered in Japan. Psicofuranine is least stable in acid (half-life 18 hours at pH 2.0 and temperature 30°) and most stable at a neutral pH (261). Studies of the kinetics of degradation and of the products formed (261–263) have yielded information of potential value for estimating stability of the compound in aqueous media and for determining conditions suitable for bioassay (264, 265). The lack of activity of psicofuranine when conventional *in vitro* bioassays are attempted has been circumvented by development of special procedures which permit determination of concentrations down to 10 mcg./ml. in water or 3 mcg./ml. in blood or serum by a plate technique or as low as 0.5 mcg./ml. turbidimetrically (264). A physical method, involving paper chromatography and ultraviolet absorption characteristics (266), and a chemical method (267) also have been published, the latter being usefully applied in studies of absorption and excretion of the antibiotic (263, 268).

Dogs excrete a fraction of orally or parenterally administered psicofuranine in the urine (263), indicating absorption. There is evidence of irreversible binding of the drug, and a tendency for it to concentrate in the kidney (269), a factor that would need to be considered in patients with renal impairment. Determination of urine levels might be a useful criterion of absorption when low doses, following which it is difficult to determine blood levels, have been administered (269).

Despite the low order of *in vitro* activity, psicofuranine "compares favorably with commercially available antibiotics" in chemotherapeutic effectiveness in animals experimentally infected with *Staphylococcus aureus* (including strains resistant to other antibiotics), *Streptococcus hemolyticus*, and *Escherichia coli* (270). There appears to be no cross resistance between psicofuranine and antibiotics in common use (270).

Oral or intraperitoneal doses of 100 mg./Kg./day effectively increase the number of survivors and tumor regressions in rats with several types of tumors (271).

Acute LD_{50} values in the mouse and rat and subacute toxicity studies in the rat and the dog indicate that psicofuranine has a relatively low order of activity for these species (271). In the latter study, there was a tendency toward leucopenia at high doses, 300 or more mg./Kg./day. At very high dosage, there may be some liver damage and loss of weight.

Ristocetin

Trade Name: Spontin (Abbott).
Biologic Origin: *Nocardia lurida*, isolated from soil collected at the Garden of the Gods, Colo.
First Reported: 1957 (272, 273).
Clinical Uses: Staphylococcal or other Gram-positive infections resistant to other antibiotics.
Dose; Adult: 2 Gm./day, in 2 or 3 divided doses, intravenously in 5% dextrose containing 250 mg. of ristocetin per 125 ml. For severe or relatively insensitive infections, doses as high as 50 mg./Kg./day (4 Gm. for 165 to 175 pound adult) or higher may be used, but maximum dose should not exceed 6 Gm./day. *Children:* Doses should be proportionately less, according to weight.
Note 1: Administration of a single normal dose by the intravenous drip should be completed in 35 to 40 minutes. If rate is slower, adequate blood and tissue levels may not be achieved; if more rapid, irritation and other undesirable effects may occur.
Note 2: Concentration of ristocetin in dextrose solution infused should not exceed 1.25%, and when use of such a concentration is necessary rate of administration should not exceed 2 ml./min.
Toxicity: Hematologic complications (anemia, neutropenia, leucopenia), rash, fever, and diarrhea have been noted. Irritation and chemical thrombophlebitis may occur if too high a concentration of drug is used.

Chemistry.—Ristocetin, as available for medicinal use, consists of a mixture of two related amphoteric compounds, ristocetins A and B. Both contain amino and phenolic groups and sugars. The molecular weights of ristocetins A and B are probably about 4,000 (273). The compounds have been isolated as free bases and crystallized as sulfates. They are soluble in acidic aqueous media, but are only slightly soluble at neutrality. They are insoluble in most organic solvents.

Ristocetin is stable in acidic aqueous solutions but is rapidly inactivated above pH 7.0. Sterile solutions at an appropriate pH can be stored in a refrigerator for at least 1 month without significant loss of potency (273).

A plate assay techique, employing an appropriate strain of *Bacillus subtilis*, and a tube-dilution assay, using a saprophytic strain of *Corynebacterium* have been developed (272). Pure ristocetin A is used as the reference standard. This fraction, which is about one-fourth to one-third as antibacterially active as ristocetin B, has been assigned an arbitrary unit value of 1,000 units/mg.

Absorption and Excretion.—No ristocetin can be detected in either the blood or the urine after oral administration, but it is absorbed after intramuscular injection and can be detected in the blood (rabbit experiments) up to 8 hours after administration of 15 mg./Kg. or for about 3 hours after an intravenous dose. Similar values have been recorded in pediatric practice (274). Currently, only the intravenous route is

employed in medicine. About 30% of an intravenous dose (in dogs) can be recovered from urine passed in the first 4 hours and an additional 15% during the next 4 hours. Glomerular filtration appears to be the principal excretion mechanism. This may be followed by some tubular reabsorption (275).

Clinical Uses.—In general, ristocetin is not as active as penicillin and some other common antibiotics against the common cocci. Therefore, and also because it must be given intravenously, it is not suitable for treatment of routine infections. However, it is of value against infections caused by some strains of *Staphylococcus* and other cocci (streptococci, enterococci, pneumococci, etc.) that are insensitive to the conventional antibiotics (276–285), although failures also have been recorded (285, 286).

Ristocetin is active against the tuberculosis organism *in vitro*, but mouse protection studies and a few clinical trials in human patients have been disappointing.

Resistance.—Initially sensitive strains of staphylococci exposed to sublethal concentrations of ristocetin *in vitro* give rise to resistant variants in a slow, stepwise pattern (272, 287), but no cases of resistance arising during clinical use have been reported. Cross resistance with other commonly used antibiotics does not seem to occur.

Toxicity.—Untoward side effects are not uncommon in patients receiving ristocetin, although if the daily dose does not exceed 25 mg./Kg. and is given in two or three equally spaced and divided doses, complications generally are minimal. Much of the effect probably depends on the concentration of the solution used and the rate at which it is infused.

The most serious effects are hematologic. Eight in a series of 10 consecutive patients given from 750 mg. in 5 days to 57 Gm. in 11 days displayed such reactions (288) including neutropenia, acute anemia and platelet depression, and leucopenia. Studies with larger numbers of patients, numbering several hundred in the aggregate, indicate neutropenia in about 4 to 7.5% of patients recieving the drug (278, 280, 289).

Ototoxicity and nephrotoxicity also have been reported to occur occasionally (285), as well as rash, and local myositis (290) and, when doses are high, diarrhea.

Thrombophlebitis and irritation are induced at the injection site if too high a concentration is used. Care must be exercised to prevent spillage into adjacent extravascular tissue to avoid irritation and, generally, intramuscular injection should be avoided. It has been reported that the inflammatory response can be decreased by addition of 5 mg. of cortisone to intramuscular doses of 25 mg./Kg. (274). This seems to be a dubious procedure, in view of the possible complications produced by steroids in infection. It seems preferable to control (or minimize incidence of) such reactions by limiting the concentration of ristocetin solution that is employed. Usually a 0.2% solution in 5% dextrose is reasonably safe if it is infused at the appropriate rate (see capsule sketch at beginning of this section). In no instance should the concentration exceed 1.25%.

Vancomycin

Trade Name: Vancocin (Lilly).
Biologic Origin: *Streptomyces orientalis* isolated from soil samples collected in Indonesia and India.
First Reported: 1956 (291, 292).
Clinical Uses: Infections due to strains of staphylococci and other Gram-positive organisms resistant to the common antibiotics.
Usual Dose; Adults: 1 to 2 Gm. daily, administered intravenously in four equally spaced and divided injections, the drug being dissolved in 5% glucose.
Children: 20 mg./lb./day, administered as above.
Toxicity: Toxicity is relatively low. Most common untoward effect is phlebitis, following repeated injections. A rash sometimes occurs and, in prolonged treatment with high doses, ototoxicity and nephrotoxicity may appear.

Chemistry.—Elemental analysis of vancomycin base indicates at least 7% nitrogen, 16 to 17% carbohydrate, and 4.37% organic chlorine. Ultraviolet and infrared analyses indicate the presence of substituted phenolic groups and hydroxyl or amino, amide, and aromatic groups. Glucose and aspartic acid moieties also are known to be present, but the complete structure has not been determined. Estimates of molecular weight have ranged from 785 to 3,500, suggesting the possibility of macromolecular aggregation. Titration data indicate a value between 3,200 to 3,500 ± 200.

The free amphoteric base is insoluble in water, but is soluble (up to about 25%) in several polar, water-miscible solvents, e.g., dimethyl sulfoxide and dimethyl acetamide. The hydrochloride and the sulfate are water soluble, the solubility of the former being in excess of 100 mg./ml. The hydrochloride is moderately soluble in aqueous methanol also. It is precipitated from aqueous solutions by heavy metals and from acidic solutions by ammonium sulfate and sodium chloride (291, 293). It is the hydrochloride which is distributed for medicinal use under the name Vancocin (Lilly).

Stability decreases abruptly below pH 3.0

and above pH 7.0 at 37°. Within that range, loss of activity is about 10% in 6 days at 37°.

At 5°, there is about 10% loss in 6 months at pH values between 1 and 9 but about the same loss in 9 days at pH 10.0.

Absorption and Excretion.—For systemic action, vancomycin must be administered parenterally. The intravenous route is preferred because of mild to moderate pain commonly accompanying intramuscular injection.

The drug permeates most body fluids quickly, following injection, but only small amounts appear in the bile and, in the absence of meningeal damage, none in the spinal fluid. After a single dose of 500 mg., in healthy adults, antibacterial concentrations of vancomycin are soon present in the blood, and assayable amounts still remain after 24 hours (294). There is some accumulation of vancomycin following multiple repeated doses, and concentrations two to three times those required to kill most strains of micrococci *in vitro* generally are achieved.

Excretion is primarily renal, and high concentrations of vancomycin occur in the urine, even after a single dose of 500 mg. Values as high as 2,400 mcg./ml. have been found 1 hour after such a dose. The average concentration after 6 hours is about 300 mcg./ml., and after 24 hours is about 100 mcg./ml. (294).

After oral administration, very little vancomycin appears in the urine and, normally, none can be found in the blood of healthy subjects but large amounts, ranging from 400 to 24,000 mcg./Gm., depending on time elapsed since administration, are found in the stools. From none to about 110 mcg./Gm. may appear in the stools after intravenous doses.

It has been concluded from experiments with animals and observations on humans that "vancomycin is not metabolized to a great extent in the body" (295).

Clinical Uses.—Vancomycin is particularly active against Gram-positive cocci, especially staphylococci (291, 296–300). Indications for its use are pneumococcal bronchopneumonia, pharyngitis, erysipelas (301), acute micrococcal endocarditis (302), and, in general, infections due to staphylococci resistant to other antibiotics (300, 303–305). Vancomycin has been called the drug of choice for oral administration in micrococcal ileocolitis ". . . because of its bactericidal effect and because of the large quantities that are excreted in the stool . . ." (306).

Impaired renal function probably is a valid contraindication for use of vancomycin systemically.

In a study of eight antistaphylococcal antibiotics, including erythromycin and novobiocin, vancomycin was rated best with respect to bactericidal action and low resistance-inducing potential (307), and in another study of 1,350 organisms, more than one-third of which were strains of *Staphylococcus aureus* resistant to penicillin, all were found to be sensitive to vancomycin (296). Others have considered vancomycin, ristocetin, and kanamycin all about equally effective clinically (285).

Resistance.—In laboratory exposure, cultures of vancomycin-sensitive bacteria may lose some sensitivity to the drug, but usually only slowly and to a small extent (294, 297, 301). Clinical experience with the drug has been excellent with respect to its low resistance-inducing potential (301, 308, 309). However, strains rendered resistant to novobiocin or to ristocetin often are resistant to vancomycin also, indicating a high degree of cross resistance among the three drugs (285).

Toxicity.—Mild to severe phlebitis may occur in 10 to 25% of patients receiving several intravenous injections of vancomycin, and a skin rash has been noted in about 4 to 5%. These are the most common side reactions. However, when high doses and/or prolonged treatment are employed (or even with normal doses if there is uremia or kidney malfunction) high blood levels may occur and under these circumstances ototoxicity has been noted (285, 302, 303, 305). But, if proper precautions are used to guard against building up too high a serum level of vancomycin, even the patient with poor renal function need not be denied this antibiotic (310).

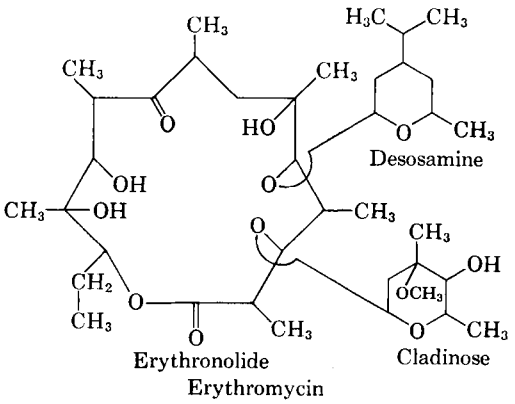
Evidence of eighth nerve damage, exhibited by auditory deficiency, generally has not been observed when serum levels have been within the limits usually provided by a total daily dose of 2 Gm. (range 7.6 to 20 mcg./ml. and average about 13 mcg./ml.) if there is no renal impairment. However, when prolonged treatment with vancomycin is indicated, serum levels of the antibiotic and renal function should be determined and audiometric tests should be made periodically in order to guard against possible deafness and nephrotoxicity.

The Erythromycin-Oleandomycin Controversy

Erythromycin, marketed by Abbott as Erythrocin and by Lilly as Ilotycin and, in the form of the propionyl ester, as Ilosone, was discovered in 1952 in culture filtrates of *Streptomyces erythreus* which had been isolated from soil collected near Iloilio City on the island of Panay in the Philippine Archipelago (311). Oleandomycin

(Matromycin, Pfizer) a metabolic product of *Streptomyces antibioticus* (312), is also available as triacetyloleandomycin (Cyclamycin, Pfizer) and as triacetyloleandomycin combined with glucosamine (Cyclamen, Wyeth; and Tao, Roerig). A 1:2 mixture of oleandomycin and tetracycline is marketed as Signemycin (Pfizer) and, phosphate-buffered, as Signemycin V (Pfizer).

Erythromycin and oleandomycin are macrolide antibiotics. Among the dozen or so other members of the macrolide group of antibiotics thus far recognized are carbomycin, griseomycin, marbomycin, methymycin, picromycin, and spiramycin. The macrolides are characterized by a highly substituted many-membered lactone system to which is attached, glycosidally, an amino sugar. Other sugar and nonsugar moieties also may be present. The chemistry of these and other antibiotics with complex ring structures, notably peptide lactones and polyene macrolides, has been reviewed (65, 66, 313). The structure of erythromycin has been determined (314), but the complete structure of oleandomycin is still unknown.



Erythromycin

Although they are active against a number of Gram-positive organisms and against selected Gram-negative species, both antibiotics are valuable primarily because of their antistaphylococcal activity, especially against strains resistant to penicillin and the tetracyclines. Both drugs are readily absorbed from the intestinal tract and are administered orally for systemic action Certain derivatives, such as the propionyl ester of erythromycin and the triacetyl-derivative of oleandomycin, are more rapidly and uniformly absorbed than the respective parent compounds and produce higher serum levels. Neither drug is significantly toxic when properly used (see reviews in the "United States Dispensatory," 25th ed., 1955 and the 1960 Supplement thereto), although rare instances of jaundiec have been attributed to prolonged use of propionyl erythromycin ester lauryl sulfate but not of other forms of erythromycin. At the clinical level, erythromycin and oleandomycin preparations may be considered strictly competitive items.

The value of erythromycin in controlling and preventing spread of epidemic penicillin-resistant staphylococcal infection in hospital nurseries has been demonstrated (315, 316), and it has been considered a satisfactory substitute for penicillin prophylaxis of group A β-hemolytic streptococcal disease (317). A study of more than 12,000 newborn infants demonstrated that erythromycin ointment (5 mg./Gm.) is equally as satisfactory as silver nitrate as a prophylactic against ophthalmia neonatorum and is much less irritating (318). Numerous reports dealing with substantial numbers of patients comment favorably also on the clinical value of oleandomycin in treating infections caused by Gram-positive organisms (319–323). There is no question that both are useful drugs. However, they cannot be considered equivalent.

Many claims for the superiority of triacetyloleandomycin have been made on the basis of the relatively high blood levels it produces, reportedly from 80 to 140% higher than oleandomycin (324) and greatly in excess of those produced by erythromycin. However, evaluations made in terms of antibacterial activity of serum rather than actual content of antibiotic reveal "no significant difference (between triacetyloleandomycin and erythromycin) . . . in the ability to inhibit bacterial growth" (325) and that the "somewhat greater (four- to fivefold) blood levels of oleandomycin did not compensate for the marked difference in antibacterial activity" (326). Erythromycin is from 4 to 10 times more active than oleandomycin against strains of pneumococci, streptococci, and staphylococci sensitive to both (326), a view supported by others (34, 42, 327). It has been pointed out that the propionyl ester of erythromycin is absorbed more consistently than the base, and that the serum levels achieved with the ester compare favorably with those attained with triacetyloleandomycin and that "in terms of antibacterial activity of the subjects' serum . . . . propionyl erythromycin gave effective serum levels that were more than 10 times as high" (326). Objective studies have led to the conclusion that "oleandomycin and spiramycin are sufficiently inferior to erythromycin to indicate that their adoption for general use in the treatment of infections is unwarranted and should be discouraged" (36, 37). Entirely apart from the question of efficacy is the matter

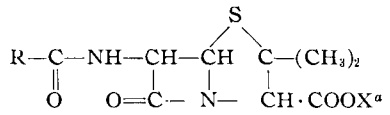
of resistance. Since cross resistance occurs between oleandomycin and erythromycin, one is faced with the situation that extensive "use of an inferior drug (oleandomycin) can deprive a better one of its usefulness" (328).

Many claims of synergism between oleandomycin and other antibiotics have been made, the most enthusiastic claims being made for a 2:1 mixture of tetracycline and oleandomycin (Signemycin, Pfizer). The view has been expressed editorially that the combination is essentially a "nostrum" foisted on an unsuspecting medical public to create a market for an "inferior agent," oleandomycin, by using it to dilute or contaminate "an established antibiotic, tetracycline," and that much of the advertising copy "exaggerates the truth and all too often only distorts it and renders it meaningless when inferior wares are being peddled" (329). When the claim for synergistic action of the mixture was shown to be misleading, advertising emphasis shifted to the claim that use of the mixture tended to retard emergence of resistant bacterial strains from initially sensitive ones. However, carefully controlled studies have failed to substantiate this claim and have shown that, after exposure to the mixture, organisms, often are resistant not only to oleandomycin but to tetracycline also, just as if they had been exposed to it alone (36, 37, 42).

Modified Penicillins

Any procedure which violates the integrity of the β-lactam or thiazolidine rings of the accompanying structure destroys the antibacterial activity of the molecule. Therefore, since the molecule is unstable under the conditions of many chemical procedures, efforts to produce biologically active new types of penicillin were seriously hampered and, until recently, the only successful attempts were those in which various precursors were added to the culture substrate with the hope that the mold would incorporate the compound or a moiety of it into the R group and produce a new form of penicillin. More than three dozen "unnatural" penicillins were biosynthesized in this way, and the structures and antibacterial activity of many of them have been summarized (4).

Synnematin B.—Synnematin, a product isolated in 1951 from *Cephalosporium salmosynnematin* (330, 331), is a mixture of two compounds (332), one of which, synnematin B, was later shown to be identical with cephalosporin N and to be a previously unknown biosynthetic penicillin, namely, D-4-amino-4-carboxy-*n*-butyl penicillin (333). Another source of synnematin B is



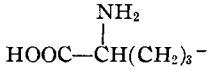
Type of Penicillin	R
Benzyl (penicillin G)	$-CH_2-C_6H_5$
D-4-Amino-4-carboxy-*n*-butyl (synnematin B, cephalosporin N)	$HOOC-CH(NH_2)(CH_2)_3-$
Phenoxymethyl (penicillin V)	$-OCH_2-C_6H_5$
Phenoxyethyl (phenethicillin)	$-O(CH_2)_2-C_6H_5$
2,6-Dimethoxyphenyl (methicillin, BRL-1241)	$-C_6H_3(OCH_3)_2$
Phenylmercaptomethyl	$-S-CH_2-C_6H_5$

[a] Depending on the type of penicillin, X in the commercially available products may be H, K, Na, or procaine.

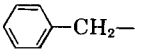
Emericellopsis terricola var. *glabra*, a member of the *Aspergillaceae* (334, 335).

Synnematin B is unique among other known penicillins because of its effectiveness against the *Salmonella* and *Proteus* groups of organisms (336–343), including typhoid and paratyphoid A infections in man (343). This activity against important Gram-negative pathogens, the low degree of toxicity (comparable to conventional penicillins), and other pharmacologically favorable characteristics (344) should engender considerable interest in this antibiotic. In a single trial, it was found curative in primary syphilis (345).

Chemical studies (330, 346, 347) and assay techniques (334, 344) have been recorded. The fact that such a pronounced change in antibacterial spectrum is produced when the



group found in synnematin B is substituted for the



group of penicillin G suggests interesting leads for further study, now that techniques for producing semisynthetic penicillins have been developed.

Semisynthetic Penicillins.—In 1957, after many years of unsuccessful attempts, Sheehan

succeeded in total synthesis of an antibacterially active penicillin (348). The product was phenoxymethyl penicillin, a compound which had been produced experimentally in the United States about ten years before by the biosynthetic technique of adding precursors to the culture substrate of the mold but had been passed over as an item for industrial production until investigators in Europe demonstrated that its acid-stability made it particularly useful for oral administration (349–353). Phenoxymethyl penicillin, better known as penicillin V, is distributed as Pen-Vee-Oral (Wyeth) and V-Cillin (Lilly).

Sheehan's work was a chemical triumph and stands as a monument to man's pertinacity in his attempts to understand and duplicate the products of nature and then to improve on them. The methods developed permitted synthesis of penicillins not previously known, but the techniques were complicated, the yields were low, and none of the compounds produced proved sufficiently superior to biosynthetic penicillins to warrant industrial production. However, it was recognized that if one of the intermediates, 6-aminopenicillanic acid, could be obtained in sufficient quantity, synthesis from that point on should not be prohibitively expensive and that the possibilities for synthesis of new compounds should be almost limitless.

British workers observed that 6-aminopenicillanic acid occurs in crude penicillin fermentation media, presumably the mold also uses this as a building block in biosynthesizing penicillin, and they found a means of stopping the fermentation at that point and removing the moiety in reasonable yield by ion exchange (354). Synthetic methods can be employed to attach various acidic and other side chains to the naturally produced 6-aminopenicillanic acid, and more than 500 such semisynthetic penicillins have now been produced (355). The first of these to be exploited commercially was the potassium salt of a mixture of the D and the L isomers of 6-(α-phenoxypropionamido)penicillinate, commonly known as phenoxyethyl penicillin and assigned the generic name phenethicillin. Others that are being studied are sodium 6-(2,6-dimethoxybenzamido)penicillinate monohydrate and phenylmercaptomethyl penicillin.

Phenethicillin is available as Alpen (Schering), Broxil (Beecham), Chemipen (Squibb), Darcil (Wyeth), Dramcillin S (White), Maxipen (Roerig), and Syncillin (Bristol). It has appeared in the literature also as PA 228, BRL 152, P 152 DL, and as penicillin 152. The dimethoxybenzamido derivative is available as Celbenin (Beecham) and Staphcillin (Bristol) and has appeared in the literature also as BRL-1241 and, more recently, as methicillin.

Synthesis and physical comparison of the diastereoisomers of phenethicillin have been reported (356). The diastereoisometric mixture is more active than the individual components against sensitive organisms and has an antibacterial spectrum similar to that of penicillin V (357). Pharmacologically (358) and in clinical practice (359, 360), the two analogs of phenethicillin appear similar. Presence of food in the upper gastrointestinal tract when phenethicillin is administered orally markedly interferes with absorption—may reduce subsequent serum levels 30 to 60%—and to ensure maximum absorption the drug should be given on a fasting stomach or at least 1 hour before meals (360), although there is some evidence that, despite the lower serum levels achieved when the drug is given after a meal, the levels that are achieved are better sustained (361).

A major advertising claim made for phenethicillin is that, when given orally, it produces much higher blood levels than penicillin V given by the same route and than penicillin G given intramuscularly. It is also claimed that phenethicillin is excreted into the urine in greater amounts than penicillin V. The advertisements fail to point out that the "greater amounts found in the urine . . . produce appreciably less antibacterial activity when assayed against the same standard," that the "higher serum levels are also less active against hemolytic streptococcus and pneumococcus and no more active against *Staph. aureus* than those produced by orally administered penicillin V" (361). Nor is it pointed out that, although the peak concentration attained with oral phenethicillin is greater than that produced by intramuscular penicillin G when assayed in terms of drug given, "the peak is much lower when compared against the same standard and expressed in terms of units of penicillin G" or that the high serum levels attained with the new product are very brief while intramuscular penicillin G produces much more sustained levels and substantially greater urinary recovery (361). It has been pointed out that "thus, although the claim of better absorption and excretion and higher serum level of phenethicillin may be partly correct, . . . this is true in a very restricted sense and is therapeutically meaningless" and actually "misleading since it clearly implies greater antibacterial and presumably curative activity, which, in fact, the drug does not possess, at least in any broad sense of the various

uses to which penicillin is put" (361). In the same study it was shown that phenylmercaptomethyl penicillin, which produces lower serum levels and is excreted in a smaller proportion in the urine provides "substantially and significantly greater antibacterial activity from equivalent doses given by mouth, fasting or after a meal, by any of the standards of comparison that were used" (361). In comparison with phenethicillin and penicillin V, phenylmercaptomethyl penicillin, whether given before or after a meal, produced the highest levels of antibacterial activity despite the fact that the concentrations in the serum were lower and a smaller proportion was recovered from the urine. As pointed out earlier in this paper, the concentration of an antibiotic in the serum is not alone a significant statistic for evaluation of its clinical value. Of equal importance is the activity of the drug against the specific pathogen to be controlled and, unless some uniform standards are applied, comparisons based on serum level, amount recovered in the urine, etc., are quite meaningless in any practical or therapeutic sense.

Others, in a comparative study of penicillins G, V, phenethicillin, and 6-(2,6-dimethoxybenzamido)penicillinate monohydrate (henceforth designated methicillin), also have drawn attention to "the quite large differences in the range of their antibacterial activity" and to the fact that "it is . . . no longer possible to treat all penicillins as though they were equivalent" (362). This situation is going to become more complicated and more confusing as the number of semisynthetic penicillins on the market multiplies unless some uniform standards of reference are adopted.

Methicillin attracts special interest because of its uniformly (to date) good performance against staphylococci resistant to penicillin G and the other penicillins currently available (362–370). It is "both stable and active in the presence of staphylococcal penicillinase" (368), although it may act as a penicillinase inducer and after 24 to 48 hours of growth of the organisms suffer some loss of activity (365). However, attempts to induce strains of *Staphylococcus aureus* to become resistant to methicillin have failed and *Staph.* strains reisolated from patients receiving the drug showed no change in the minimum inhibitory concentration (365). A novel proposal for controlling staphylococcal cross infection in hospitals is the spraying of methicillin into the air to produce droplets which circulate much as the staphylococci do. It has been reported that such a procedure prevented the usual acquisition of pyogenic staphylococci by newborn infants (371).

Methicillin is unstable in acid media; therefore, the oral route is not suitable for systemic medication. Given slowly by deep intramuscular injection, it is no more painful than penicillin G and no local reactions at the injection site have been noted. A dose of 1 Gm. of the sodium salt at 4- to 6-hour intervals has been recommended for adults (368).

A good comparative appraisal of the newer penicillins has been published by Dowling (371a).

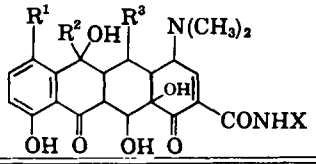
Modified Tetracyclines

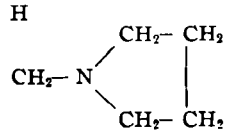
Despite the similarity of structure of members of the tetracycline family listed in the accompanying generalized formula, they are not quantitatively identical antimicrobially or pharmacologically. For example, frequently CTC is more active against staphylococci and pneumococci, OTC against *Pseudomonas pyocyanea*, and TC against *Proteus* (372, 373). The differences may be slight or relatively large, depending on the species and strain of organism involved. However, there is complete cross resistance among the three compounds, whether the organisms are resistant when isolated from clinical material (344) or are induced by exposure to any one of them in the laboratory (375).

Pharmacologic differences are somewhat greater, especially with respect to absorption and excretion and to gastrointestinal side effects. TC generally produces higher and more prolonged antibiotic activity than OTC and CTC in the blood (376), and it is less likely to induce severe gastrointestinal disturbance, especially the acute, and sometimes fatal, staphylococcal enterocolitis which may follow treatment with CTC or especially OTC (377–380). Principally because of its supposed pharmacologic advantages, TC has largely replaced CTC and OTC in clinical practice.

Demethylchlortetracycline (Declomycin, Lederle).—The demethylchlortetracyclines, originally produced in 1957 by chemical manipulation of TC (381–383), constitute a group of derivatives with greater acid- and alkali-stability than the corresponding methylated compounds. Subsequently, 6-demethyltetracycline and 7-chloro-6-demethyltetracycline (DMCT) were found among the metabolic products of a mutant strain of *Streptomyces auriofaciens*, the organism from which CTC was obtained.

One of these, DMCT (available as Declomycin, Lederle), was found to be, on the average, about twice as active as TC against many pathogens and rarely less active, to be readily absorbed following oral administration, and to be excreted



Drug	Symbol	R^1	R^2	R^3	X	Year Discovered
Chlortetracycline	CTC	Cl	CH_3	H	H	1948
Oxytetracycline	OTC	H	CH_3	OH	H	1950
Tetracycline	TC	H	CH_3	H	H	1953
Demethylchlortetracycline	DMCT	Cl	H	H	H	1957
N-(Pyrrolidinomethyl)tetracycline	PMT	H	CH_3	H		1958

about half as rapidly as TC. A thorough and authoritative review of the bacteriologic, clinical, and pharmacologic position of DMCT has been published (384).

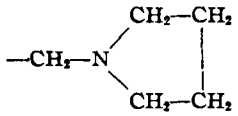
Against some strains of streptococci, DMCT may be almost 5 times as active as TC, although the average figure is about 2.3 (385). Against some resistant staphylococci, it may show somewhat lesser relative activity (386).

Presumably because of the lower rate of excretion of DMCT, therapeutic serum levels are maintained for a longer time after administration of this antibiotic than after an equivalent dose of TC. Therefore, satisfactory therapy can be achieved with DMCT given in smaller, or in less frequent, doses. DMCT, like its analogs, is concentrated in the liver and excreted into the bile, where the concentration may be up to 30 times that occurring in the serum at the same time (387).

Toxicity of DMCT appears to be no greater than that of its earlier analogs. In moderate doses, its tendency to cause diarrhea is minimal, and in therapeutically equivalent doses DMCT probably tends to produce slightly less, certainly not more, gastrointestinal side effect than TC.

A curious phototoxicity not observed in patients receiving other TC analogs has occurred in some subjects exposed to bright sunlight while under treatment with DMCT. The reaction manifests itself as an exaggerated sunburn with high fever, eosinophilia, and increased blood platelets. The effect occurs only on skin exposed to rays in the 2,700 to 3,200 Å. range, which is filtered out by ordinary window glass, and appears to be a true phototoxicity, not merely a hypersensitivity phenomenon (384, 388-390). The incidence is not definitely established. One observer noted 4 cases among 27 ambulatory patients (389) while another reported 40 instances among 2,682 patients (390).

Pyrrolidinomethyltetracycline (Syntetrin, Bristol). — N - Pyrrolidinomethyltetracycline (PMT) is prepared, by partial synthesis, from TC by replacing one hydrogen of the NH_2 group of the latter with



The dry compound is reasonably stable—loss of potency 15% in 4 months at 56°, 18% in 1 year at 37°, and 7% in 18 months at 25° (391). In solution, it may retain full potency for 1 or 2 days at room temperature. The compound is soluble in water, and concentrations of 1 Gm./ml. can be prepared over the pH range 1.5 to 8.5. Its chemical and physical properties have been reviewed (392) and abstracted (393).

Qualitatively and quantitatively, PMT is similar to TC in antibiotic spectrum. The principal advantage claimed for the derivative over the parent compound is greater solubility in the physiologic pH range (394). For systemic use it is given parenterally, and it may be a useful addition to the armamentarium for the patient who needs tetracycline therapy but cannot (or will not) accept oral medication. It is claimed to provide higher blood levels than TC and to cause less pain and irritation at the injection site (391), and to provide better therapeutic effects and fewer side effects, especially gastrointestinal, than oral TC (395–397). It is interesting that, despite the claim of less pain at the injection site, in preparing an intramuscular dosage form of PMT a local anesthetic (lidocaine, 40 mg./350 mg. PMT) has been added. Both the intramuscular and the intravenous dosage forms contain ascorbic acid.

One intravenous injection of 275 mg. per day for 4 to 7 days is normally adequate for average

infections due to sensitive organisms. Severe infections or those caused by less sensitive strains may require 2 to 4 injections daily. In children, 5 to 25 mg./Kg. is the normal dose range.

Intramuscular doses are given by deep injection into the upper outer quadrant of the gluteal muscle. Injection into dermal or subcutaneous tissue must be avoided. Injection in such tissues may cause unnecessary pain and tissue reaction. In intravenous administration, extravenous spillage must be avoided for the same reason.

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Research Articles

Synthesis and Pharmacological Studies of Some Aliphatic Hemicholinium Analogs

By MARVIN F. POWERS, STEFAN KRUGER, and FRED W. SCHUELER

The aromatic nucleus of hemicholinium-3 was replaced with an aliphatic hexamethylene chain without loss of the characteristic pharmacological activity. The toxic dose was elevated in mice about tenfold by the change. Choline chloride was a very effective antidote to intoxication from the aliphatic hemicholinium. Another compound synthesized, a pyridine analog, had marked anticholinesterase activity, but caused a flaccid paralysis in avian musculature. A third compound, a trimethylamine analog, elicited neuromuscular blockade typical of decamethonium.

HEMICHOLINIUM-3, hereafter called HC-3, was synthesized in 1954 during an investigation of a series of bis-quaternary ammonium compounds (1). The general pharmacology of HC-3 has recently been reviewed by Schueler (2). Marshall and Long (3) synthesized and investigated pharmacologically some hemicholinium analogs and found none to be more potent than HC-3. The analogs studied by these workers contained structural changes at the cationic heads and in the biphenyl nucleus. It appears that the crucial moiety is at the cationic head rather than at the biphenyl grouping, since the introduction of an ether or a methylene linkage between the two phenyls elicited only a slight decrease in potency, whereas certain relatively minor changes at the cationic heads either markedly reduced or abolished HC-3-like activity (3). Considering the alterations already studied, one may question whether the aromatic nucleus is an essential feature in the HC-3 molecule. The introduction of a methylene chain would not only incorporate an aliphatic moiety, but would also provide an approach for a systematic study of the effect on pharmacologic activity

Received March 11, 1961, from the Department of Pharmacology, Tulane University Medical School, New Orleans 12, La.
Accepted for publication May 17, 1961.
Supported in part by U.S.P.H.S. grant.
Taken in part from a thesis submitted to the Graduate School, Tulane University, in partial fulfillment for the degree, Master of Science in Pharmacology.

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By MARVIN F. POWERS, STEFAN KRUGER, and FRED W. SCHUELER

The aromatic nucleus of hemicholinium-3 was replaced with an aliphatic hexamethylene chain without loss of the characteristic pharmacological activity. The toxic dose was elevated in mice about tenfold by the change. Choline chloride was a very effective antidote to intoxication from the aliphatic hemicholinium. Another compound synthesized, a pyridine analog, had marked anticholinesterase activity, but caused a flaccid paralysis in avian musculature. A third compound, a trimethylamine analog, elicited neuromuscular blockade typical of decamethonium.

HEMICHOLINIUM-3, hereafter called HC-3, was synthesized in 1954 during an investigation of a series of bis-quaternary ammonium compounds (1). The general pharmacology of HC-3 has recently been reviewed by Schueler (2). Marshall and Long (3) synthesized and investigated pharmacologically some hemicholinium analogs and found none to be more potent than HC-3. The analogs studied by these workers contained structural changes at the cationic heads and in the biphenyl nucleus. It appears that the crucial moiety is at the cationic head rather than at the biphenyl grouping, since the introduction of an ether or a methylene linkage between the two phenyls elicited only a slight decrease in potency, whereas certain relatively minor changes at the cationic heads either markedly reduced or abolished HC-3-like activity (3). Considering the alterations already studied, one may question whether the aromatic nucleus is an essential feature in the HC-3 molecule. The introduction of a methylene chain would not only incorporate an aliphatic moiety, but would also provide an approach for a systematic study of the effect on pharmacologic activity

Received March 11, 1961, from the Department of Pharmacology, Tulane University Medical School, New Orleans 12, La.
Accepted for publication May 17, 1961.
Supported in part by U.S.P.H.S. grant.
Taken in part from a thesis submitted to the Graduate School, Tulane University, in partial fulfillment for the degree, Master of Science in Pharmacology.

of alteration of the distance between the cationic heads, provided an aliphatic chain can successfully replace the biphenyl nucleus, with retention of characteristic hemicholinium-like action.

The aliphatic moiety least likely to alter extended molecular length would seem to be a hexamethylene chain, since in its extended configuration this chain approximates very closely the length of the biphenyl nucleus. The aromatic nucleus is rigid and permits no variation of distance between cationic heads, whereas the methylene chain allows a great deal of folding. However, the presence of two positively charged atoms in this molecule would necessarily entail coulombic repulsion between the like charges and thus tend to cause the molecule to be in a fully extended configuration. This paper reports the pharmacological studies of some aliphatic HC-3 analogs containing a hexamethylene chain.

MATERIALS AND METHODS

Suberic acid (0.1 mole) was reacted with 0.3 mole of thionyl chloride at 50° for 4 hours in a flask fitted with a reflux condenser (4). The excess thionyl chloride was then evaporated under reduced pressure. The acid halide was added dropwise to an ethereal solution of diazomethane at 0°. The diazomethane was prepared from Diazald[1] and dried over potassium hydroxide for 30 minutes. The reaction mixture was allowed to stand at room temperature overnight. The precipitated bis-diazoketone was then dissolved in chloroform, and anhydrous hydrochloric acid was bubbled through the solution for 20 minutes. Evaporation of the solvent left the crude compound 1,6-bis(chloroacetyl)-*n*-hexane as a brown mass.

After four recrystallizations from aqueous methanol, the product was obtained in the form of white plates melting at 85°, as stated in the literature (4), with a 60% yield.

One gram of the 1,6-bis(chloroacetyl)-*n*-hexane was dissolved in 15 ml. of cold dioxane, mixed with an excess (2 ml.) of β-dimethylamino ethanol, and heated on a water bath for 30 minutes. The gummy material which formed was dissolved in absolute alcohol. Upon addition of ether a white solid separated out. Three recrystallizations gave a product melting at 212°, with decomposition. This product, 1,10-bis(dimethyl-β-hydroxyethylammonium)-*n*-decan-2,9-dione dichloride was designated P-16-A.

The pyridine compound, P-16-B, was prepared in a similar manner, using pyridine as the amine. This product melted at 216°, with decomposition.

P-16-C was prepared by reacting trimethylamine with the 1,6-bis(chloroacetyl)-*n*-hexane in a pressure bottle for 2 hours at 100°. The product melted at 225°, with decomposition.

The melting points in all cases involving decomposition were determined by placing the compound on a hot Fisher-Johns melting block heated to within a few degrees of the expected melting point. Analyses of these compounds are detailed in Table I. Figure 1 shows the molecular configurations of these compounds and also that of HC-3.

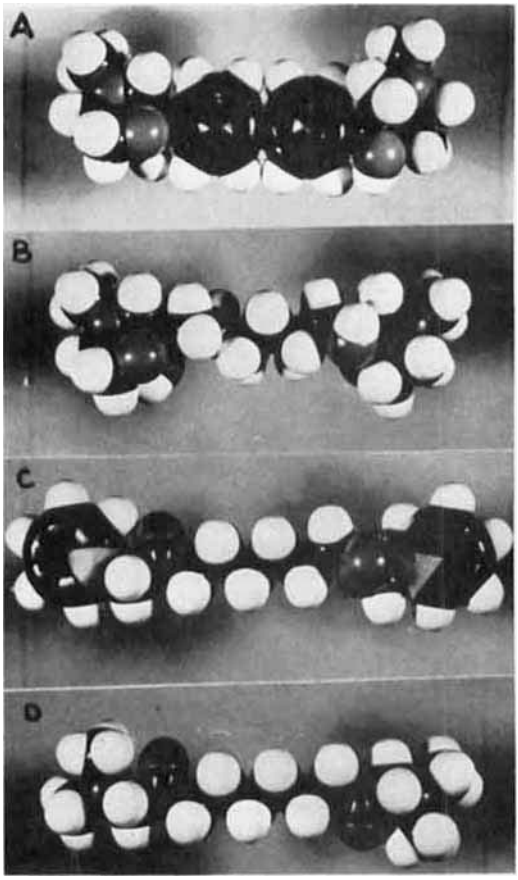


Fig. 1.—It can be seen that internitrogen distances are similar when the molecules are maximally extended. A is HC-3, B is P-16-A, C is P-16-B, and D is P-16-C.

Animal Studies.—Toxicity studies were done on white Swiss mice weighing about 25 Gm. In each case the compound was dissolved in 0.9% sodium chloride and injected intraperitoneally. Each dose level was administered to a group of 10 mice. Toxicity data were calculated by the method of Litchfield and Wilcoxon (5). Drug antagonism studies were carried out by administering various drugs in conjunction with each of the compounds being investigated, as specified in Table II.

Studies on blood pressure, sciatic-gastrocnemius activity, and respiratory function were done on nine mongrel dogs anesthetized with sodium pentobarbital 35 mg./Kg. intravenously. The trachea was cannulated and a water manometer was attached for recording respiration on smoked kymograph paper. The left common carotid artery was cannulated and blood pressure recorded by using a mercury manometer. Five milliliters of 2% mepesulfate (Roche) was introduced into the carotid cannula to avoid clotting of blood. The right vagus nerve was isolated and sectioned, and periodically the periph-

[1] Aldrich Chemical Co.

TABLE I.—TOXICITY, MELTING POINT, AND ANALYSIS OF COMPOUNDS

Compound	M. P., °C.	LD_{50} in Mice, mg./Kg.	Elemental Analysis, % Required C	Required H	Required N	Found C	Found H	Found N
P-16-A $C_{18}H_{38}Cl_2N_2O_4$	212	0.5 (0.38–0.65)	51.7	9.1	6.7	51.6	9.0	6.7
P-16-B $C_{26}H_{26}Cl_2N_2O_2$	216	10.0 (6.7–13.0)	59.1	6.7	6.8	59.3	6.8	6.5
P-16-C $C_{16}H_{34}Cl_2N_2O_2$	225	6.5 (5.8–7.2)	53.7	9.5	7.8	53.5	9.2	7.5

TABLE II.—PHARMACOLOGICAL TESTS OF COMPOUNDS

Situation	No. Mice	Compound	Dose mg./Kg.	Route	Compd. Used Jointly	Dose, mg./Kg.	Route	Deaths
1	10	P-16-A	0.5	i.p.	....	..	...	9
2	10	P-16-A	0.5	i.p.	Choline	20	s.c.	2
3	10	P-16-A	1.0	i.p.	Choline	20	s.c.	9
4	10	P-16-A	1.0	i.p.	Choline	40	s.c.	5
5	10	P-16-B	12.0	i.p.	....	..	...	9
6	10	P-16-B	12.0	i.p.	Choline	20	s.c.	8
7	10	P-16-B	12.0	i.p.	Atropine	2.0	s.c.	7
8	10	P-16-C	7.0	i.p.	....	..	...	10
9	10	P-16-C	7.0	i.p.	Choline	20	s.c.	8
10	10	...	..	..	Choline	40	s.c.	0

Chi-Square

Situation	P
1 and 2	<0.01
3 and 4	>0.10
4 and 5	>0.50

eral stump was electrically stimulated. The right sciatic nerve was isolated, ligated tightly, and arranged for supramaximal peripheral stimulation with a Grass model S4C stimulator at specified frequencies by shielded silver electrodes. The gastrocnemius was also isolated, sectioned distally, and connected to a lever for kymographic recording. Test doses of histamine, epinephrine, and acetylcholine were administered several times to obtain a standard response. The vagus was stimulated, and the intact right carotid artery was temporarily occluded for additional standard responses. This testing operation was conducted periodically after administration of P-16-A, P-16-B, and P-16-C to screen for autonomic-cardiovascular effects.

Bilateral sciatic-gastrocnemius preparations were done on 12 adult albino rabbits anesthetized with 200 mg./Kg. of phenobarbital sodium administered intravenously 1 hour prior to the experiment. Both sciatic nerves were tightly ligated and individually arranged for supramaximal peripheral stimulation by a Grass stimulator at various frequencies, using shielded silver electrodes. The gastrocnemius muscles were attached to kymographic levers for recording contraction height.

Seven chicks, four weeks old, were injected intramuscularly with 1 or 2 mg. of compound and observed for evidence of flaccid or spastic paralysis.

Twelve adult white leghorn hens were anesthetized with 25 mg./Kg. of sodium pentobarbital and prepared for kymographic recording by isolating the sciatic nerve, ligating it, and arranging it for peripheral stimulation supramaximally at specified frequencies at 5 msec. duration. The gastrocnemius muscle was attached to a kymograph lever. The cannulated trachea was insufflated with oxygen.

Enzyme Studies.—Enzyme inhibition studies were carried out manometrically, using "true" acetylcholinesterase, a commercial purified preparation of bovine erythrocyte acetylcholinesterase.[2] For the competitive studies, "pseudo" cholinesterase was used; the source was fresh dog plasma diluted 1:5 with 0.9% sodium chloride. Flasks contained a total volume of 2.0 ml. for each experiment. The standard procedure of Ammon was used as modified by Augustinsson (6).

Acetylcholine was used as the substrate, and a bicarbonate buffer served as the medium. All appropriate flask readings were corrected for nonenzymatic hydrolysis and for atmospheric pressure variations. Readings were taken every 10 minutes for a period of 1 hour in the experiments using "true" esterase, and every 5 minutes for a 40-minute period in the "pseudo" esterase experiments. Results were expressed as μl. of carbon dioxide evolved per interval of time. Determinations were always carried out using duplicate flasks.

RESULTS AND DISCUSSION

The toxicity of the aliphatic hemicholinium, P-16-A, is not as great as that of HC-3, the former being about one-tenth as potent in mice. See Table I. This decrease might be explained if one assumes that not all the molecules are maximally extended and that complete extension is essential for optimal activity. Similarly, the toxicities of the pyridine and trimethylamine analogs were decreased by about the same degree.

Figure 2 shows a typical result of P-16-A on a

[2] Winthrop Laboratories.

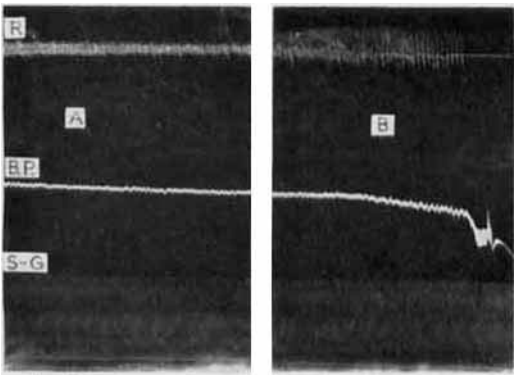


Fig. 2.—A recording of dog respiration (R), blood pressure (B.P.), and sciatic-gastrocnemius (S-G) effects when P-16-A, 1 mg./Kg. is administered at A. Respiratory function failed at B, 105 minutes after A, whereas the S-G activity continued.

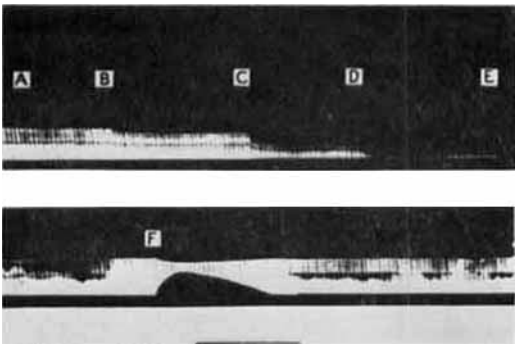


Fig. 3.—Adult hen sciatic-gastrocnemius preparation and the effects of P-16-B (top record) given in graded doses. A = 0.5 mg./Kg.; B = 1.0 mg./Kg.; C = 1.25 mg./Kg.; D = 1.5 mg./Kg.; E = 2.00 mg./Kg. No contracture is evident even though P-16-B is a cholinesterase inhibitor in mammals. Bottom record is P-16-C 80 mcg./Kg. given at F.

dog. The neuromuscular responses continue unaltered when stimulation is one per 10 seconds. However, the respiration fails about 100 minutes after administration. This latent action is very typical of HC-3 poisoning. Blood pressure falls only after respiratory embarrassment and failure have occurred. If oxygen is insufflated intratracheally throughout respiratory paralysis, the blood pressure remains stable, and eventually the respiratory function is restored. Administration of choline chloride intravenously, 20 mg./Kg., antagonizes the respiratory paralysis.

In a rabbit sciatic-gastrocnemius preparation P-16-A mimics HC-3. Frequency of stimulation is an important factor, since a frequency rate of one per 10 seconds is never accompanied by failure or by depression of function in the dosage used. In contrast, the rate of one per second is accompanied by functional failure. It is indeed true that an increase of frequency of stimulation can lower the effective dose of a neuromuscular blocking agent such as curare and decamethonium (7). However, with the latter two, it was observed that onset was rapid.

The trimethylamine analog (P-16-C) did not produce an HC-3-like response. The onset of paralysis was rapid and was not antidoted by choline chloride, as was P-16-A. See Table II. The functional response of the nerve-muscle preparation was actually further depressed by choline chloride. The hen sciatic-gastrocnemius preparation exhibited a mixed response with depolarization of end-plates, as reflected by contracture, and a slight flaccid paralysis, as represented by the decrease in contraction height. See Fig. 3.

The pyridine analog, P-16-B, exhibited effects which were very interesting. When 1 mg./Kg. was administered to dogs, a transient fall in blood pressure was noted; when a test dose of acetylcholine was given, the recovery of blood pressure was delayed. Increasing the dose of P-16-B to 5 mg./Kg. caused neuromuscular paralysis. Both doses caused copious salivation, micturition, and defecation. These latter effects are also observed in the intact, unanesthetized rabbit. Even though the electrical stimulus was predetermined to have been supramaximal, a transient increase in contraction height was observed.

A similar effect is observed with well-known cholinesterase inhibitors such as eserine. When the present compounds were studied in the presence of cholinesterase, it was found that P-16-B was the most potent *in vitro* inhibitor of the three compounds synthesized (see Table III). Assuming that cholinesterase inhibition was the primary action of P-16-B, it would be expected that the compound would produce depolarization and spastic paralysis in the chicken. The actual effect observed is, however, a flaccid paralysis (see Fig. 4). That P-16-B does not produce even transient spasticity is demonstrated in Fig. 4. When one starts with a very small dose P-16-B (1 mcg./Kg.) and gradually increases the dosage in a hen sciatic-gastrocnemius preparation, there is no apparent effect until a dose of 1 mg./Kg. is given. This latter dose causes a decrease in contraction height with no evidence of contracture. Table III illustrates the type of enzyme inhibition that P-16-B seems to produce. It was found that inhibition is apparently competitive in nature. Plotting the reciprocal of activity at several concentrations of the inhibitor (P-16-B) against the reciprocal of substrate concentration yields "curves" which share the same origin on the ordinate, thereby

TABLE III.—STUDIES OF CHOLINESTERASE ACTIVITY BY MANOMETRIC METHODS[a]

Acetylcholinesterase from Bovine Erythrocytes, Concentration		
Compound	Moles	% Inhibition
HC-3	1×10^{-3}	25 (1)[b]
P-16-A	1×10^{-3}	68 (1)
	1×10^{-4}	19 (2)
P-16-B	1×10^{-3}	97 (1)
	1×10^{-4}	93 (1)
	1×10^{-5}	78 (1)
	1×10^{-6}	33 (1)
P-16-C	1×10^{-3}	53 (1)
	1×10^{-4}	29 (1)
Eserine	1×10^{-3}	96 (1)
	1×10^{-4}	96 (1)
	1×10^{-5}	91 (1)
	1×10^{-6}	67 (1)

[a] Acetylcholine concentration, 1×10^{-3} *M*.
[b] Figures in parentheses represent number of determinations using duplicate flasks.

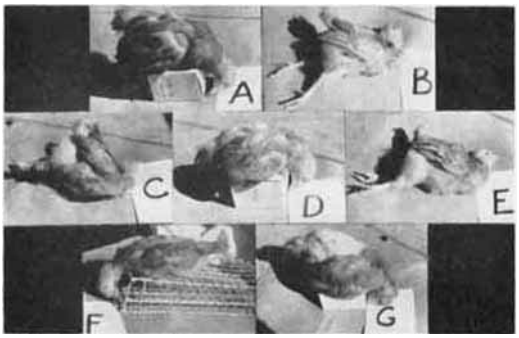


Fig. 4.—Four-weeks old chicks injected intramuscularly with the corresponding compound. Dosage was 1 mg. total dose for each. A = Curare; B = decamethonium; C = succinylcholine; D = HC-3; E = P-16-C; F = P-16-B; G = P-16-A. All compounds caused rapid paralysis within 5 minutes except HC-3 (D) and P-16-A (G) which produced paralysis after a latent period of 20 to 30 minutes.

resembling the Lineweaver-Burk plot of a competitive inhibitor.

The fact that P-16-B, a potent anticholinesterase agent, effects a flaccid rather than a spastic type of paralysis in avian muscle is particularly interesting. A possible explanation may follow from the concepts of Ariëns, Van Rossum, and Simonis (8). P-16-B may represent a product with very high affinity but low intrinsic or specific activity with respect to postsynaptic acetylcholine receptors at the neuromuscular junction. Thus, while P-16-B is a potent anticholinesterase drug, its high affinity (in the manner of curare) causes it to block the actions of excess acetylcholine at this junction. For muscarinic receptors P-16-B apparently does not exhibit such a high affinity and therefore the effects of excessive acetylcholine due to its anticholinesterase effects are observed.

SUMMARY

Three new substances related to hemicholinium were synthesized: 1,10-bis(dimethyl-β-hydroxyethyl ammonium)-*n*-decane-2,9-dione dichloride, 1,10-bis(pyridinium)-*n*-decane-2,9-dione dichloride, and 1,10-bis(trimethylammonium)-*n*-decane-2,9-dione dichloride.

Pharmacological studies indicated that the biphenyl nucleus of HC-3 can be replaced with an aliphatic chain without serious loss of HC-3-type activity as observed by the actions which P-16-A produced. Possible reasons for the decrease in toxicity of the compounds have been discussed.

One of the compounds, P-16-B, was an anticholinesterase agent nearly as potent as eserine. Paradoxically, this compound prevented endplate depolarization. A possible explanation concerning the flaccid paralysis is discussed.

P-16-C appeared to exert a mixed action by causing contracture paralysis (spasticity), accompanied by a slight decrease in contraction height in a chicken sciatic-gastrocnemius preparation.

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Substituted Sulfonyl Piperazines

By WINTHROP E. LANGE, ROBERT N. LAZARUS, and MERLE E. AMUNDSON†

In view of reports in the literature on the anticonvulsant action of various sulfonyl piperazines and of certain substituted 1-sulfonyl-4-β-aminoethylpiperazines as antishock agents, a series of mono- and disulfonyl β-aminoethylpiperazines have been prepared. The compounds were obtained, in most cases, by a modified Schotten-Bauman reaction. The carbobenzyloxy blocking group was employed in the preparation of the monosubstituted compounds and was removed by catalytic hydrogenation without cleavage of the sulfonyl group.

A LARGE NUMBER of 1,4-unsymmetrically substituted sulfonyl piperazines has been prepared for various purposes. Among them are 1-ethylsulfonyl-4-ethylpiperazine (1), a compound active in the treatment of hemorrhagic or heat shock, and 1-diethylsulfonyl-4-(2′-thiazoyl)piperazine (2), active as an anticonvulsant. Jacob and Suau (3) later found that 1-substituted sulfonyl-4-β-aminoethylpiperazines were useful in the treatment of hemorrhagic and traumatic shock. The presence of a β-aminoethyl group in other piperazine compounds has, in general, provided compounds with pharmacological activity, varying from adrenergic (4) to curarimimetic (5). This agrees with many of the known antihistamines, antispasmodics, and local anesthetics in which the ethylene group between oxygen and nitrogen or nitrogen and nitrogen provides the most active compounds. This suggested that the activity of sulfonyl piperazines could possibly be enhanced by inserting a β-aminoethyl group between the sulfonyl group and the piperazine nucleus. It also appeared desirable to prepare 1-β-aminoethylpiperazines containing two sulfonyl groups. The present investigation is intended to make available for testing various arylsulfonyl compounds.

DISCUSSION

The methods which have been employed in the preparation of the disubstituted arylsulfonyl compounds have generally involved the treatment of 1-β-aminoethylpiperazine with two equivalents of an arylsulfonyl chloride in aqueous media with an inorganic base to neutralize the liberated hydrogen chloride, or in alcoholic media with pyridine as the hydrogen chloride acceptor. The second procedure was more time-consuming and did not give good yields. The products, which are described in Table I, were obtained in a good state of purity either as the free base or as the sodium salt. They also gave correct carbon-hydrogen analyses after one recrystallization. It was noted that the carbon-hydrogen analyses for 1-benzenesulfonyl-4-β-benzenesulfonamidoethylpiperazine also would be the same for the monosulfonated compound and differentiation was not possible by treating the compound with dilute acid. However, the calculated nitrogen content of the two compounds was different. The product obtained gave a correct nitrogen analysis which proved that it was disubstituted.

It was also decided to prepare the monosulfonated β-aminoethylpiperazines, i.e., 1-arylsulfonyl-4-β-aminoethylpiperazine. It is necessary, therefore, to have a blocking group present on one of the nitrogens of the piperazine ring, prior to the introduction of the β-aminoethyl group, which can be removed by a nonhydrolytic procedure.

The blocking group selected for investigation was carbobenzyloxy, since it may be cleaved from the piperazine nitrogen by catalytic hydrogenation. 1-Carbobenzyloxypiperazine was prepared by a method based on that of Goldman (6), which in turn was modified by Foye and Fedor (7). The amino group of β-aminoethyl chloride, in turn, was protected with a benzoyl group prior to its reaction with 1-carbobenzyloxypiperazine. 1-Carbobenzyloxy - 4 - β - benzamidoethylpiperazine was readily prepared and the blocking group was removed by catalytic hydrogenation in a manner similar to the one reported by Goldman and Williams (8). The hydrogenation was successfully carried out using hydrogen and 10% palladium-on-charcoal in refluxing alcohol.

The desired 1-arylsulfonyl-4-β-benzamidoethylpiperazine was obtained by shaking the 1-β-benzamidoethylpiperazine with the appropriate arylsulfonyl chloride in water with sodium hydroxide or sodium carbonate to neutralize the liberated hydrogen chloride. Hydrolysis of the benzoyl blocking group with acid also cleaved the arylsulfonyl group. The compounds obtained in this manner are shown in Table I. The equation illustrates the synthesis of 1-benzenesulfonyl-4-β-benzamidoethylpiperazine.

For comparative purposes, several new arylsulfonylpiperazine compounds were prepared. When a basic aqueous-alcoholic solution of 1-carbobenzyloxypiperazine was treated with various arylsulfonyl chlorides at room temperature a good yield, in most cases, of 1-carbobenzyloxy-4-arylsulfonylpiperazines resulted. Hydrogenation by the previously described procedure gave the 1-arylsulfonylpiperazines shown in Table II. When the catalytic hydrogenation procedure was employed for the removal of the carbobenzyloxy group from 1-

Received August 16, 1960, from the Massachusetts College of Pharmacy, Boston, and the Division of Pharmacy, South Dakota State College, Brookings.
Accepted for publication October 6, 1960.
Presented to the Scientific Section, A.PH.A., Washington, D. C., meeting, August 1960.
† Fellow of the American Foundation for Pharmaceutical Education, 1959–1960.
The authors are indebted to Dr. N. Sperber of the Schering Research Laboratories for carrying out and providing the results of a pharmacodynamic screening of the compounds.

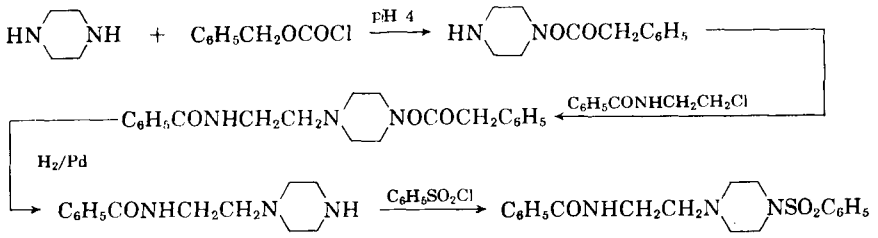
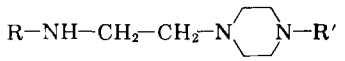


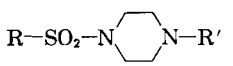
TABLE I.—SUBSTITUTED β-AMINOETHYL PIPERAZINES



R	R'	M.p.,[a] °C.	Formula	Analyses, % Calcd.	Found[b]
Benzoyl	CBzO[c]	104–105	$C_{21}H_{25}N_3O_3$	C, 68.65	68.86
				H, 6.86	6.88
Benzoyl	Benzenesulfonyl	235–239	$C_{19}H_{23}N_3O_3S$	C, 61.09	60.85
				H, 6.21	6.01
Benzoyl	*p*-Nitrobenzene sulfonyl	210–212	$C_{19}H_{22}N_4O_5S$	C, 54.69	54.85
				H, 5.28	5.65
p-Nitrobenzene-sulfonyl	*p*-Nitrobenzene-sulfonyl	207–209	$C_{18}H_{21}N_5O_8S_2$	C, 43.29	43.00
				H, 4.24	4.14
p-Aminobenzene-sulfonyl	*p*-Aminobenzene-sulfonyl	206–207	$C_{18}H_{25}N_5O_4S_2$	C, 49.18	49.07
				H, 5.73	5.87
p-Toluenesulfonyl	*p*-Toluenesulfonyl	160–162	$C_{20}H_{27}N_3O_4S_2$	C, 54.90	55.12
				H, 6.22	6.29
Benzenesulfonyl	Benzenesulfonyl	144–146	$C_{18}H_{23}N_3O_4S_2$	C, 52.86	52.90
				H, 5.67	5.73
				N, 10.27	9.95

[a] The melting points were taken on a Fisher-Johns block and are uncorrected. [b] The analyses were carried out by Weiler and Strauss, Oxford, England, and by Geller Laboratories, Bardonia, N. Y. [c] CBzO = carbobenzyloxy.

TABLE II.—SUBSTITUTED SULFONYL PIPERAZINES



R	R'	M.p.,[a] °C.	Formula	Analyses, % Calcd.	Found[b]
p-Toluene	CBzO[c]	145–147	$C_{19}H_{22}N_2O_4S$	C, 60.93	60.72
				H, 5.92	5.89
p-Toluene	H	103–105	$C_{11}H_{16}N_2O_2S$	C, 55.00	54.76
				H, 6.72	6.50
p-Toluene	Benzyl	121–123	$C_{18}H_{22}N_2O_2S$	C, 65.45	65.66
				H, 6.71	7.05
p-Nitrobenzene	CBzO	155–156	$C_{18}H_{19}N_3O_6S$	C, 53.33	53.41
				H, 4.73	4.75
p-Aminobenzene	H	210–213	$C_{10}H_{15}N_3O_2S$	C, 49.99	50.39
				H, 6.23	6.25

[a, b, c] See footnotes, Table I.

carbobenzyloxy - 4 - *p* - nitrobenzenesulfonylpiperazine it was found, as expected, that the nitro group was also reduced to an amino group. In one case the arylsulfonylpiperazine was further treated with α-chlorotoluene to give the 1-arylsulfonyl-4-benzylpiperazine.

Antishock and anticonvulsant tests have not been completed with these compounds. However, two of the compounds, 1-β-benzenesulfonamidoethyl-4-benzenesulfonylpiperazine, and 1-β-(*p*-aminobenzenesulfonamidoethyl)- 4- *p* - aminobenzenesulfonylpiperazine were inactive in a general pharmacodynamic screen and central nervous system test carried out at the Schering Research Laboratories.

EXPERIMENTAL

Melting points were taken on a Fisher-Johns block and are uncorrected. Analyses were obtained from the Weiler and Strauss Microanalytical Laboratory, Oxford, England, and from Geller Laboratories, Bardonia, N. Y.

N-Benzoyl-β-chloroethylamine.—This compound was prepared by the procedure of Brintzinger, Pfannstiel, and Daddebusch (9). An 85% yield of white needles was obtained which melted at 105–106° as previously reported (9).

1-Carbobenzyloxypiperazine.—This compound was prepared by the procedure of Goldman and

Williams (6). A 45% yield of colorless product was obtained which boiled at 160–165° (2 mm.). The reported boiling point (6) is 158–161° (1.4 mm.).

1 - Carbobenzyloxy - 4 - β - benzamidoethylpiperazine.—A mixture of N-benzoyl-β-chloroethylamine (5.5 Gm., 0.03 mole), sodium carbonate (12 Gm.), and 1-carbobenzyloxypiperazine (6.6 Gm., 0.03 mole) in xylene (100 ml.) was refluxed for 6 hours or until carbon dioxide no longer was evolved from the reaction mixture. The excess sodium carbonate and the sodium chloride formed during the reaction were removed by filtration, and the xylene was removed by vacuum distillation. The residue was recrystallized from isopropyl alcohol to yield 9.8 Gm. (89%) of 1-carbobenzyloxy-4-β-benzamidoethylpiperazine; m.p. 104–105°.

1 - Benzenesulfonyl - 4 - β - benzamidoethylpiperazine.—In a 300-ml. three-neck flask equipped with a hydrogen inlet tube, a mechanical stirrer, and a reflux condenser was placed 3.7 Gm. (0.01 mole) of 1 - carbobenzyloxy - 4 - β - benzamidoethylpiperazine and 1 Gm. of 10% palladium-on-charcoal in 100 ml. of ethanol. The mixture was stirred and heated by an oil bath at 85° while hydrogen was bubbled in continuously. After 6 hours, when the evolution of carbon dioxide had ceased, the catalyst was removed by filtration and the solvent was evaporated under reduced pressure. A chilled aqueous solution of the residue was treated with 1.8 Gm. (0.01 mole) of benzenesulfonyl chloride and 4.0 ml. of 4 *N* sodium hydroxide. After stirring for 3 hours at room temperature, the mixture was cooled and filtered, yielding 2.1 Gm. (57%) of white crystals. The product was first recrystallized from water and then from ethanol to give white needles melting at 235–239°.

1 - *p* - Nitrobenzenesulfonyl - 4 - β - benzamidoethylpiperazine.—The hydrogenation procedure was identical with the one described for the previous preparation. A chilled aqueous solution of the residue was treated with 2.2 Gm. (0.01 mole) of *p*-nitrobenzenesulfonyl chloride and 4.0 ml. of 4 *N* sodium hydroxide. After stirring for 6 hours at room temperature, the mixture was cooled and concentrated under reduced pressure. The product was recrystallized from an alcohol-water mixture to yield 1.8 Gm. (64%) of pale yellow crystals; m.p. 210–212°.

1 - *p* - Toluenesulfonyl - 4 - carbobenzyloxypiperazine.—To a solution of 10.24 Gm. (0.04 mole) of 1-carbobenzyloxypiperazine and 7.7 Gm. (0.04 mole) of *p*-toluenesulfonyl chloride in 100 ml. of an ethanol-water mixture was added a few pellets of sodium hydroxide. The mixture was shaken for 3 hours, allowed to stand overnight, and the white precipitate was collected. Recrystallization from ethanol yielded 5.1 Gm. (34%) of 1-*p*-toluenesulfonyl - 4 - carbobenzyloxypiperazine; m.p. 145–147°.

1-*p*-Toluenesulfonylpiperazine.—A solution containing 3.74 Gm. (0.01 mole) of 1-*p*-toluenesulfonyl-4-carbobenzyloxypiperazine in ethanol was hydrogenated in an analogous manner to that previously described. The product obtained after evaporation of the alcohol was recrystallized from a methanol-water mixture. A yield of 1.3 Gm. (54%) of 1-*p*-toluenesulfonylpiperazine was obtained; m.p. 103–105°.

1 - *p* - Toluenesulfonyl - 4 - benzylpiperazine.—A mixture of 1-*p*-toluenesulfonylpiperazine (1.2 Gm., 0.005 mole), sodium carbonate (1 Gm.), and α-chlorotoluene (0.63 Gm., 0.005 mole) in xylene (50 ml.) was refluxed for 6 hours. The excess sodium carbonate and the sodium chloride were removed by filtration, and the xylene evaporated under reduced pressure. Recrystallization from methanol yielded 1.2 Gm. (75%) of pale brown platelets melting at 121–123°.

1 - *p* - Nitrobenzenesulfonyl - 4 - carbobenzyloxypiperazine.—To a solution of 10.24 Gm. (0.04 mole) of 1-carbobenzyloxypiperazine and 8.86 Gm. (0.04 mole) of *p*-nitrobenzenesulfonyl chloride in 100 ml. of ethanol was added a few pellets of sodium hydroxide. A small amount of heat was applied to the mixture to initiate the reaction. The mixture was shaken for 2 hours, and after refrigeration overnight, the white precipitate was collected. Recrystallization from ethanol yielded 14.3 Gm. (96%) of 1 - *p* - nitrobenzenesulfonyl - 4 - carbobenzyloxypiperazine; m.p. 155–156°.

1-*p*-Aminobenzenesulfonylpiperazine.—A solution containing 6 Gm. (0.015 mole) of 1-*p*-nitrobenzenesulfonyl-4-carbobenzyloxypiperazine in ethanol was hydrogenated in an analogous manner to that already described. The product obtained after evaporation of the solvent was recrystallized from ethanol. A yield of 3 Gm. (75%) of product was obtained which melted at 210–213°.

1 - β - (*p* - Nitrobenzenesulfonamidoethyl) - 4 - *p*-nitrobenzenesulfonylpiperazine.—To 6.43 Gm. (0.05 mole) of 1-β-aminoethylpiperazine[1] and 22.2 Gm (0.1 mole) of *p*-nitrobenzenesulfonyl chloride in 100 ml. of water was added sufficient sodium hydroxide to maintain an alkaline pH. The mixture was stirred for 8 hours, acidified with dilute hydrochloric acid, and the precipitate collected. Recrystallization from an ethanol-acetone mixture yielded 13 Gm. (52%) of pale yellow needles which melted at 207–209° (decompn.).

1 - β - (*p* - Aminobenzenesulfonamidoethyl) - 4-*p*-aminobenzenesulfonylpiperazine.—To a solution of 10 Gm. of stannous chloride dihydrate in 10 ml. of concentrated hydrochloric acid was slowly added 2.5 Gm. (0.005 mole) of 1-β-(*p*-nitrobenzenesulfonamidoethyl) - 4 - *p* - nitrobenzenesulfonylpiperazine. The reaction temperature was kept at 25–30° by cooling with water. After the addition was complete, and the reaction mixture had been stirred for 2 hours at room temperature, it was poured into a cold solution of 4 *N* sodium hydroxide. The resulting white precipitate was collected on a filter and recrystallized from an ethanol-water mixture. A yield of 1.8 Gm. (82%) of crystalline solid was obtained which melted at 206–207°.

1 - β - (*p* - Toluenesulfonamidoethyl) - 4 - *p*-toluenesulfonylpiperazine.—A solution of 5 Gm. (0.04 mole) of β-aminoethylpiperazine, 19 Gm. (0.1 mole) of *p*-toluenesulfonyl chloride, 100 ml. of an ethanol-water mixture, and 4 Gm. of sodium hydroxide was shaken for 3 hours at room temperature. The mixture was allowed to stand overnight at room temperature, and after cooling it was acidified with dilute hydrochloric acid. The resulting white solid was collected, dried, and recrystallized from ethanol, yielding 7.3 Gm. (43%) of product; m.p. 160–162°.

[1] Aldrich Chemical Co.

The monohydrated sodium salt of 1-β-(*p*-toluenesulfonamidoethyl) - 4 - *p* - toluenesulfonylpiperazine was obtained by filtration of the precipitate which appeared in the reaction mixture before acidification; m.p. 213-215°.

Anal.—Calcd. for $C_{20}H_{23}N_4NaO_5S_2 \cdot H_2O$: C, 50.20; H, 5.90. Found: C, 49.5; H, 5.9.

1 - β - Benzenesulfonamidoethyl - 4 - benzenesulfonylpiperazine.—This compound was prepared in a manner similar to that described in the previous procedure. A yield of 89% of colorless needles was obtained which melted at 144–146°.

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Prediction of Stability in Pharmaceutical Preparations VIII

Oil-in-Water Emulsion Stability and the Analytical Ultracentrifuge

By EDWARD R. GARRETT†

An oil-in-water emulsion clears in a centrifugal field to form a packed, semirigid "cream." Subsequently, a true oil or continuous phase is spun-out of the "cream." It is shown that rates of emulsion clearing can be stated as a function of the ultracentrifugal r.p.m. and that the rate of clearing under simple gravity can be predicted for the oil-water emulsion as initially constituted. "Bad" emulsion can be differentiated from "good" emulsion by the ready formation of a separate oil phase in the former. In the latter case, an induction period exists before a continuous oil phase is spun out at an accelerating rate, which rate is a function of the centrifugal force.

THE PREDICTION of stability in pharmaceutical preparations has largely dealt with chemical transformations (1, 2). The basic philosophy has been the application of stress or energy to promote decomposition and the following of degradation or of some property of the degradation as a function of time. This function has been linearized and the slopes of such linear plots have been used as estimates of specific rates (k). The reproducibility of such functions at varying degrees of stress would validate their use in prediction of rates under marketing or storage conditions. There are classical relations that describe the variation of such rate parameters with the degree of stress. In the cases previously discussed, a rate at a given temperature is linearized on the basis of it being first, second, etc., order and the apparent rate constant (k) or property proportional to rate constant is correlated with the absolute temperature by means of the Arrhenius expression, which in its logarithmic form is

$$\log k = -(\Delta H_a/2.303\ R)(1/T) + \log P \qquad \text{(Eq. 1)}$$

Thus the accelerated reactions can be used to evaluate the parameters of Eq. 1, i.e., ΔH_a and the resultant quantitative expression may be used to predict specific rates or functions thereof at lower temperatures.

Since the nature of the function is known from the reproducibility among the various accelerated studies, the predicted rate constants may be used in such a function to predict thermal stability at lower temperatures.

The same philosophy may be used in predicting physicochemical stability of pharmaceutical formulations where preservation of the pharmaceutical elegance is of primary interest rather than the integrity of the drug.

A case in point is a colloidal suspension or emulsion. An admirable example of an oil-water emulsion[1] (3, 4) is available commercially. It is a

Received March 27, 1961, from The Research Division, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication June 15, 1961.
Presented in part at Third Annual Industrial Pharmaceutical Research Conference, Land-O-Lakes, Wis., June 1961.
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clinically useful fat emulsion in an aqueous continuous phase which is well tolerated on intravenous administration and has therapeutic utility (4) since it is a high caloric material for intravenous alimentation.

The primary criteria for the stability of the preparation is the preservation of the emulsion or the retention of the complete dispersion of fat in water. A classical evaluation of such stability is vigorous shaking for 72 hours at 5° with essentially no change in particle size. Particle size may be estimated from hemocytometer particle counts. It has been shown that well over 90% of the fat is present in particles which were submicroscopic with not over 0.02% of the visible particles larger than 1.5 μ in diameter (3).

The importance of the maintenance of stability of such a preparation is obvious. Not only would nondispersed oil be conducive to embolism, but unstable oil particles would resist metabolism where the particulate nature of the fat is considered mandatory. Solvolytic degradation would tend to decrease emulsion stability. Phosphatide hydrolysis would diminish dispersing agent and increase solvolysis rates. Such a model of degradation would be autocatalytic in nature.

There are two aspects of the stability prediction of colloidal suspensions or emulsions. The one relates to the shelf life of the emulsion when gravitational influences alone are to be considered, i.e., oil flotation of the emulsion.[1] The other is the stability prediction of emulsion when chemical degradation produces an inherent emulsion instability or tendency for oil coalescence. This paper primarily reports on studies of the former or physical instability, but also considers the implications of the latter.

EXPERIMENTAL

Analytical Ultracentrifuge.—The data presented in this paper were obtained from quantitative measurement of photographs of sector cells filled with the oil-water emulsion.[1] The sector cell was counterbalanced in the rotor of the Spinco analytical ultracentrifuge, model E (5) which may be operated at speeds up to 60,000 r.p.m. The optics are a Schlieren optical system (5, 6) but may also be used to measure light transmittance in different levels of the cell. The operating temperature was maintained within 2° of the initial temperature of 29° for the duration of a run.

The oil-water emulsion[1] was prepared as specified in the literature (3, 4). It was opaque and initially the photographic film was sensitized only by light which passed through (*a*) transparent air above the emulsion, i.e., toward the center of the rotor, and (*b*) a reference hole in the counterbalance. This latter is manifested in the photographic negative by a dark reference bar from which the distance to the center of the rotor is 7.30 cm. (see Figs. 1b and 5b).

The clearing of the oil-water emulsion on ultracentrifugation was demonstrated by an increasing transparency in the bottom of the cell, i.e., away from the center of the rotor. The recording of this phenomenon on the photographic negative which magnifies the actual cell 2.21 times radially (or along the vertical length in the figures) was by an increasing band of darkness. The difference between the distance, x in cm. corrected for magnification, of the leading edge of this band to the near edge of the reference bar and 7.30 cm. is the distance of the slowest floating oil particles from the center of the rotor (Fig. 5b). The measurements of the x distance were performed in two ways: (*a*) by magnification in a photo enlarger and projection on a system of coordinates with a selection of scale such that 10 times the magnification of the original cell geometry resulted, and (*b*) by use of the Spinco Analytrol to determine the optical absorbance of the photographic film as a function of the distance across the film (Fig. 6b). This latter technique gave a good sharp line at the water-emulsion or water-cream interphase. Both methods gave data in good agreement.

Specific Emulsions.—Freshly prepared emulsion,[1] maintained at 5°, preparation A, and a similar material, preparation B, maintained for one-half year at 37°, were ultracentrifuged at speeds ranging from 12,590 to 52,640 r.p.m. and the clearing of the emulsions was measured (see Figs. 1–6). The log $(7.30 - x)$ cm. values (Fig. 5b) were plotted against time for each r.p.m. and the constant slopes, k, were determined. These values are listed in Table I.

The preparation B was considered as an example of unsatisfactory emulsion in that its acidity had increased; it gave grossly observable oil and significant changes in particle size when subjected to vigorous shaking for 72 hours at 5°.

The flotation rates of oil particles were extreme at the higher r.p.m. values. Thus, most of the maximum possible clearing of the emulsion had occurred in the time necessary to bring the ultracentrifuge up to the desired r.p.m. setting. Since the valid flotation rate for a given r.p.m. could only be estimated from measurements made at constant r.p.m., the largest error of estimate of k is at the larger r.p.m. values.

TABLE I.—SLOPES, k, OF PLOTS OF LOGARITHM OF POSITION OF CLEARING EMULSION FROM CENTER OF ROTOR IN CM. AGAINST TIME IN SECONDS FOR VARIOUS R.P.M. OF ULTRACENTRIFUGATION

R.P.M.	Prepn. A[a]	Prepn. B[b]
12,590	2.34,[c] 2.35[d]	2.39
15,220	3.96, 3.68	3.67
20,410	5.95	6.03
24,630	7.21	6.53
29,500	9.31	9.67
35,600	13.4	16.9
39,460	19.8	17.9
44,770	31.8	...
52,640	39.1	...

[a] "Good" emulsion. [b] "Bad" emulsion. [c] After the "cream" formed, i.e., when no change in emulsion clearing was observed with time, material was resuspended using a Burrell wrist-action shaker at high speed for 20 minutes. [d] k Values obtained for resuspended material described in footnote[c].

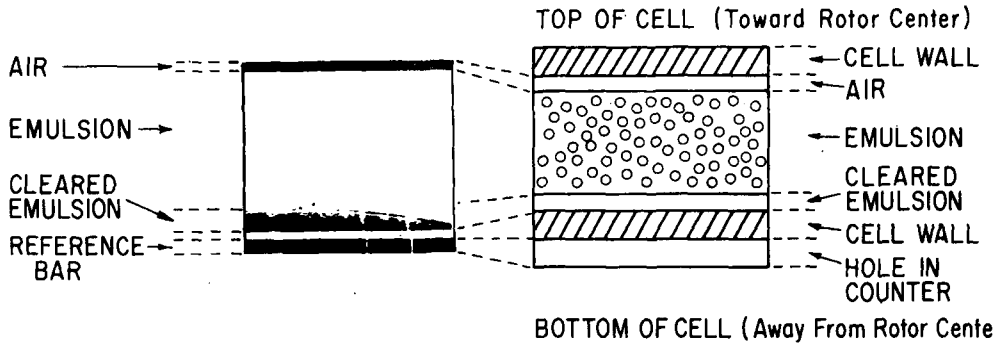


FIG. 1a
338 sec.

FIG. 1b



FIG. 2
559 sec.

FIG. 3
779 sec.

FIG. 4
1,039 sec.

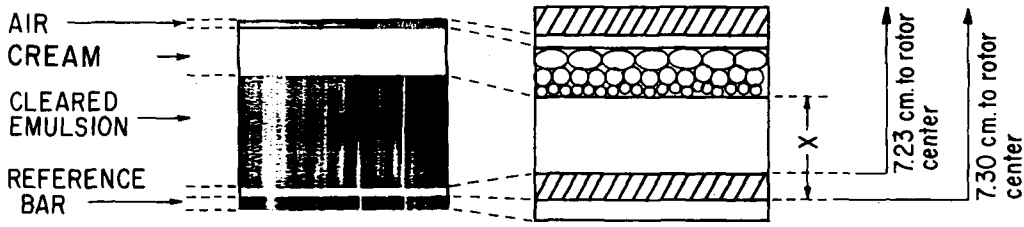
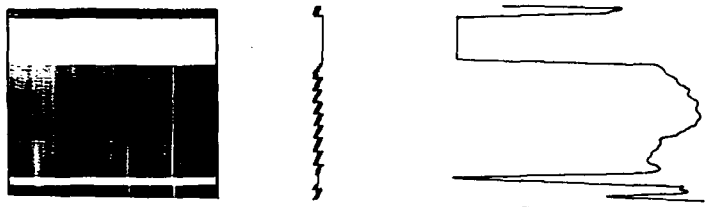


FIG. 5a
1,931 sec.

FIG. 5b



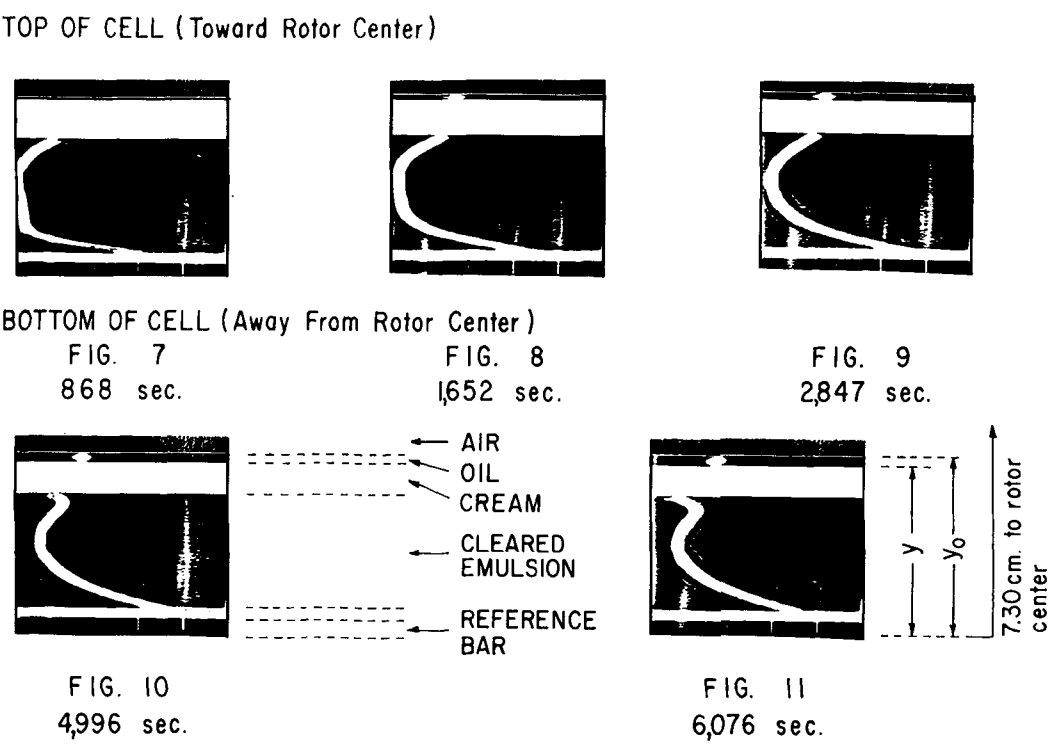
3,001 sec.
FIG. 6a

ABSORBANCE
FIG. 6b

Figs. 1a–6b.—Clearing of oil-water emulsion at 24,630 r.p.m. The ultracentrifugal pictures were taken at 2.21 magnification of the i.v. emulsion at 32°. The rotor reached full speed at 158 seconds. The times in seconds of ultracentrifugation are given below the appropriate figures. Figs. 1b and 5b interpret the photographs as to the clearing of the emulsion to the flotation of oil particles as a "cream," Fig. 6b shows a plot of the absorbance of the film.

Inspection of the material in the sector cell after ultracentrifugation showed a clear liquid at the bottom and a resilient white creamy froth or "cream" at the top of the cell with semisolid or plastic attributes which readily redispersed in the aqueous phase on agitation to give the same k constant as before (see Table I).

The formation of an oil phase occurred at higher r.p.m. values (see Figs. 7–16). Runs were conducted on both preparations A and B from 39,460 to 59,780



Figs. 7–11.—Flotation of oil in a "poor" oil-water emulsion at 52,640 r.p.m. from the "cream" of adhered oil particles. The times in seconds of ultracentrifugation are given below the appropriate figures. Figs. 10 and 11 are labeled for identification of the layers. The geometry of cell with respect to the center of the rotor is given in Fig. 11. The rotor reached full speed in 6 minutes.

r.p.m., and the depth of the oil phase was correlated with the time of ultracentrifugation (see Fig. 11 for $y - y_0$). The nature of the oil phase was demonstrated by its immiscibility with water and miscibility with oil.

Thermal instability was checked by subjection of emulsion[1] A to 75° and removal of aliquots on the third and subsequent days. These samples were toxic and on gross observations showed poor dispersivity and high acid titer. On ultracentrifugation at 52,640 r.p.m. no band on the photographic negative appeared that could be assigned to formation of an oil layer (see Figs. 10 and 11). However, inspection of the cell after centrifugation did show that the oil had separated but was now opaque.

CALCULATIONS AND RESULTS

Sedimentation of particles heavier than the suspending medium and flotation of particles lighter than the suspending medium follow the same laws on ultracentrifugation except for sign. The former sediment away from the axis of rotation; the latter float toward the axis of rotation. The theory of convection-free sedimentation in centrifugal fields as developed by Svedberg (5–7) applies to a sector-shaped cell whose side walls are in the direction of the centrifugal force so as to provide an unobstructed path to the floating particles.

The classical expression relating the distance, x, of a particle from the axis of rotation with the time, t, of centrifugation and the r.p.m. of the rotation is

$$\log x_2/x_1 = \frac{V(\rho - \rho')\ (\text{r.p.m.}2\pi)^2\ (t_2 - t_1)}{k\eta} \qquad \text{(Eq. 2)}$$

where x_2 and x_1 are positions of the particle at t_2 and t_1, respectively, V is the volume of the particle, ρ and ρ' are the densities of the particle and medium, respectively, k is a shape constant for the particle size, and η is the coefficient of viscosity of the fluid.

For a given material at a given temperature, the equation reduces to

$$\log x_2 = kt + \log x_1 = S(\text{r.p.m.})^2 t + \log x_1 \qquad \text{(Eq. 3)}$$

where t is the time from the start of the centrifugation and S is defined as the sedimentation (or flotation) constant.

It is apparent that a plot of the logarithm of the position of a floating particle from the axis of rotation against the time of ultracentrifugation should be a straight line. Typical photographs of emulsion clearing as characterized by the appearance of a nonopaque vehicle are given as Figs. 1 through 6. The value 7.30-x cm. represents the position of the slowest floating particle from the axis of rotation. The plots of the logarithm of this value for a given r.p.m. against time are straight lines as shown by the examples in Fig. 17. These plots confirm the validity of Eqs. 2 and 3.

It also follows that plots of the slope $k = S(\text{r.p.m.})^2$ against the square of the r.p.m. should be linear and

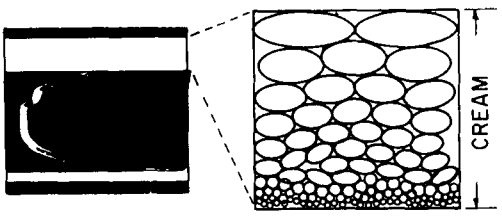


FIG. 12a
2,130 sec.

FIG. 12b

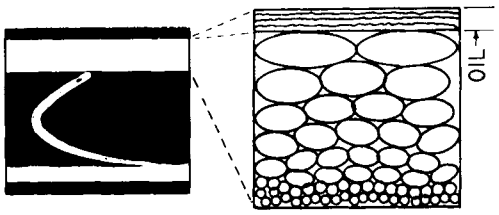


FIG. 13a
6,626 sec.

FIG. 13b

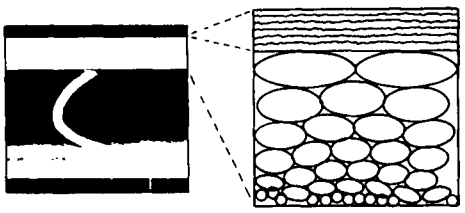


FIG. 14a
12,601 sec.

FIG. 14b

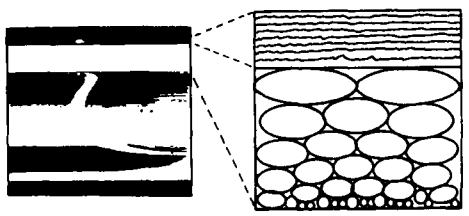


FIG. 15a
21,628 sec.

FIG. 15b

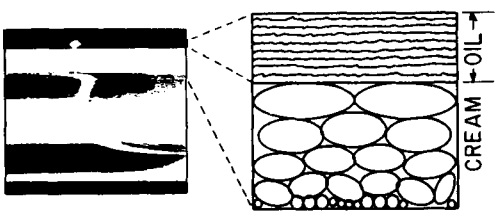


FIG. 16a
25,500 sec.

FIG. 16b

Figs. 12a–16b.—Flotation of oil in a "good" oil-water emulsion, i.v. emulsion, at 52,640 r.p.m. from the "cream" of adhered oil particles. The times in seconds of ultracentrifugation are given below the appropriate *a* figures. The *b* figures are diagrammatic representations of the coalescence of particles in the "cream." The rotor reached full speed in 6 minutes.

pass through the origin as do the examples given in Fig. 18. The resultant sedimentation coefficient is $S = 12.14 \times 10^{-14}$ for rate of rotation in min.$^{-1}$ and rate of particle displacement in the cell in sec.$^{-1}$ as calculated from the slope of the line in Fig. 18.

The data plotted in Fig. 18 are given in Table I and are typical of the data obtained from both "good" and "bad" emulsions, defined as such by the stability tests previously described. Maintenance of emulsion[1] at 40° for one-half year produces a "bad" emulsion.

The observed clearing of the emulsion by ultracentrifugation does not vary with the degree of "goodness" of the emulsion. This is not wholly unexpected since an oil-in-water emulsion should posses a spectrum of oil particle sizes and, the smaller the particle, the greater its resistance to flotation by Brownian motion and charge repulsion, i.e., higher charge/mass ratio.

In general, flotation, creaming, and coalescence are not necessarily correlated with emulsion clearing as manifested by the appearance of a clear solution. It follows that a "bad" emulsion may have a sufficient population of small oil particles which impart turbidity to the solution for as long a time as "good" emulsion under the same conditions of ultracentrifugation. It also follows that the measurement of the emulsion clearing rate and the sedimentation constant measures only the flotation of that population of particles which is the smallest size possible and says nothing about the population of larger particles.

The clearing of "good" emulsion[1] proceeded until a narrow band of opaque material remained at the "top" of the cell, adjacent to the air-liquid boundary, i.e., closest to the axis of rotation (see Figs. 5a and 5b). A clear liquid slowly appeared adjacent to the air-liquid boundary and in both "bad" (Figs. 7–11) and "good" (Figs. 12a–16b) emulsions replaced a portion of the area of opacity. The initial rate of clearing of the opaque band for "good" emulsion[1] accelerated with time, as can be observed by the plots of Fig. 19, curves b, c, and d.

Inspection of the sector cell after ultracentrifugation disclosed a clear aqueous solution below a semisolid froth-like band or "cream" which lay below a transparent oil. Agitation of the cell redispersed the "cream" which had the same flotation parameter for emulsion clearing as did new or fresh emulsion.

Emulsion which was characterized as "bad" on the premises of poor dispersivity, high acid titer, and toxicity did not show the long inductive period of "good" emulsion in spinning out oil from the "cream" (see Fig. 19a). Whereas the "good" emul-

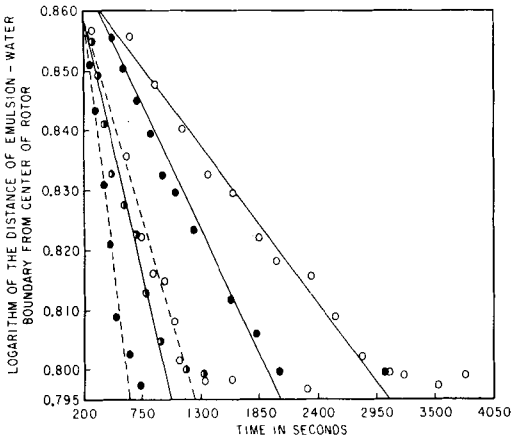


Fig. 17.—Examples of clearing of oil-water emulsion from i.v. emulsion at various centrifugal speeds. Plots of the logarithm of the distance of the emulsion-water boundary from the center of the rotor against the time of ultracentrifugation in seconds at 30°. The symbols and corresponding r.p.m. are: —⊙—, 12,590; —●—, 15,220; - -O- -, 20,410; —◑—, 24,630; - -●- -, 35,600.

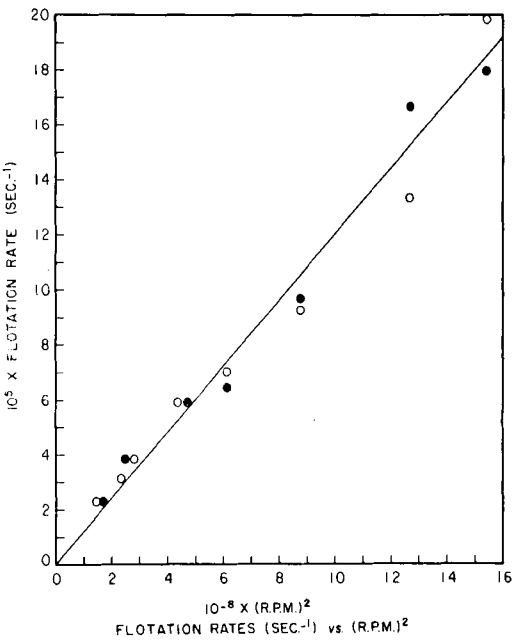


Fig. 18.—Flotation rates for the clearing of oil-water emulsion at 30° against the square of the r.p.m. The open circles are for a stable or "good" emulsion. The closed circles are for an unstable or "poor" emulsion. The flotation rates are the slopes of the plots of the logarithm of the distance of the emulsion-water boundary from the rotor center against time for each r.p.m. The apparent flotation constant from the slope of this figure is 12.14×10^{-14}.

sion manifested an induction period and then an acceleration of rate in spinning out oil, where such factors are an apparent function of r.p.m. or centrifugal field intensity (see Figs. 19b, c, and d), "bad" emulsion produced oil almost instantly with a first-order decay in amount of oil formed. Subsequent to this initial spin-out by "bad" emulsion the inductive and accelerated process of further oil spin-out characteristic of "good" emulsion was noted (see Fig. 19a).

The readily spun-out oil of "bad" emulsion had an apparent half-life in this particular sector cell of 1,200 seconds and was relatively independent of the r.p.m. values studied. However, all the emulsion was well cleared by that time for the same r.p.m. For as low as 35,600 r.p.m. the emulsion was cleared in 620 seconds. When the determined S value is used in Eq. 3, it can be calculated that the emulsion would be cleared in less than 200 seconds at 59,600 r.p.m.

It is thus apparent that the rate of coalescence to produce an oil layer, even with "bad" emulsion, was significantly slower than the rate of clearing of the oil emulsion of the smallest oil particle.

DISCUSSION

The coincidence of the apparent flotation rates of "good" and "bad" oil-in-water emulsions indicated that a population of the smallest oil particles was present in both preparations and lagged behind on ultracentrifugation. An alternative explanation is that the solutions were so concentrated that the larger oil particles "swept out" the emulsion, that is, aggregates were formed during the process of emulsion clearing that had similar clearing rates in both "good" and "bad" emulsions. This latter explanation implies that there would be no gradation in size of oil particles from bottom to top of the clearing emulsion and thus in the ultimate "cream." It also suggests that Eq. 2 would not hold, that there would be a nonlinearity early in the plots of Fig. 17 as

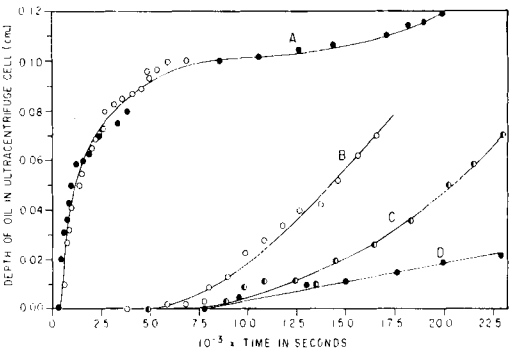


Fig. 19.—Increase of oil coalescence as a function of time for "bad", curve A, and "good", curves B, C, and D, oil-water emulsions. The symbols and ultracentrifugal r.p.m. at 30° are: ⊙, 59,780; ◐, 52,640; ●, 47,660.

aggreates were formed by "sweep-out." However, no deviation from linearity was observed in such plots until the emulsion clearing approached the meniscus or the phase boundary of the "cream."

Also, the slower the ultracentrifugal speeds, the greater would be the opportunity for particle "slippage" and thus gradation according to size. If aggregation on ultracentrifugation (before "cream" formation) was a distinct factor, the flotation rates

would not be expected to be as linear with the (r.p.m.)^2 as they were in Fig. 18.

It may also be predicted that clearing rates would be expected to change with the dilution of the oil-in-water emulsion by the continuous (water) phase if it is the aggregates rather than the smallest particles that determine emulsion clearing rates since particle stratification would be more favored under dilute conditions. Investigations are planned to evaluate these possible models.

The flotation of oil particles to the top of the cell continued until a "cream" of oil particles was formed, i.e., an adhered mass of particulate oil where the "skin" of each particle is highly resistant to the applied gravitational forces.

A model can be proposed that is consistent with the facts to explain the coalescence of the "cream."

In general, the larger oil particles should be on top of the small (see Fig. 12b). The higher the oil concentration, the less probable should be a truly systematic gradation of particle size. On subjection to gravitational force the larger particles are more readily deformed than the smaller in the same manner that large balls of mercury on a table top are more deformed than small ones (8). The larger particles are thus more subject to unequal forces and the skin may be more readily ruptured so that coalescence of two larger particles may occur to form even larger ones (see Figs. 13b–16b). The strength of the "skin" would be a function of the dispersing agent concentration and the retention of maximum conditions in the vehicle for its function and stability.

In the case of "bad" emulsion, the production of higher acidity most probably by hydrolysis indicated a variation in conditions which decreased the skin-strength of the more readily deformed larger particles. The coalescence, which undoubtedly proceeded to a minor degree in the nonstressed "poor" emulsion, was thus accelerated in the "cream" where maximum close contact of oil particles was effected. Sufficient numbers of unstable large particles exist in "bad" emulsion so that after a definite amount of distortion (since the rate of coalescence is very much less than the rate of creaming) coalescence readily occurred as a function of the numbers of large unstable particles.

Subsequent to this spin-out of oil by a first-order process under gravitational stress, the phenomenon encountered in "good" emulsion becomes predominant. In this case, the skins of the larger particles had greater strength than those in "bad" emulsion. Nevertheless, they coalesced to form larger particles but a certain population had to exist before a continuous phase of oil appeared. Simultaneously, the populations of smaller particles were coalescing although at lower rates. However, since the greatest portion of the oil is distributed among the populations of small particles and since these need more time to coalesce, an acceleration in the formation of a continuous oil phase was noted only after a large induction period.

Since the force of deformation is a function of r.p.m. (see Appendix), it is to be expected that the rate of this acceleration of continuous oil phase formation would also be a function of the r.p.m.

After the ready coalescence of the relatively large, unstable, deformed particles in "bad" emulsion, only a fraction of the total oil available had been spun out. The residual small oil particles followed the same coalescing pattern of the "good" emulsion.

This model is consistent with the prediction that the stability of a rigid sphere approaching an unbounded plane is inversely proportional to the radius of the sphere, provided that electrical double layer interaction, electroviscuous effects and London-van der Waals forces of attraction are negligible (9). Rigid behavior was reported to occur with high interfacial tensions, small bubble diameters (<0.1 cm.), and in the presence of surfactants, conditions which certainly exist in our oil-in-water emulsions (9, 10). In an oil/water system containing surfactants, Cockbain and McRoberts (10) have shown that the stability of oil droplets increased rapidly with decreasing diameter below 0.1 cm.

These studies and the conclusions drawn therefrom provide the necessary background for the prediction of stability of emulsions and dispersions by use of the analytical ultracentrifuge.

Knowledge of flotation parameters under the stress of a centrifugal field permits the prediction of flotation (or sedimentation) under the influence of simple gravity. The Appendix provides a detailed procedure which when applied to the emulsion[1] predicts that less than 1 cm. depth of the oil-water emulsion as initially constituted would be cleared in a year's time. It can also be safely stated that no significant coalescence of the oil particles in the initially constituted emulsion to form a continuous phase would occur during the pharmaceutical lifetime of the product.

The specific qualification considered is that the preparation is as initially constituted. Obviously, when chemical changes occur with time and result in "bad" emulsion, this is not so.

However, the effect of chemical deterioration on emulsion stability can be studied in such a manner as to expedite prediction. Ultracentrifugal characterization of thermally stressed preparations would permit evaluation of rate constants of continuous oil phase appearance as functions of gravitational stress. Measurements of sedimentation and coalescent rates as functions of enhanced temperature and time of subjection to enhanced temperature should permit prediction of emulsion stability by combining the lessons learned from accelerated thermal and ultracentrifugal stability studies.

SUMMARY

1. It is shown that the analytical ultracentrifuge is an excellent tool for the evaluation of emulsion stability.

2. The slopes of the plots of the position of the emulsion-water boundary from the center of the ultracentrifugal rotor against time are shown to be functions of the square of the r.p.m.

3. The discontinuous oil phase is cleared from the continuous phase by ultracentrifigation to form a cream, a packed particulate mass of adhered oil globules. The rate of clearing under simple gravity has been predicted for the emulsion as initially constituted. Less than 1 cm. depth

of the oil-water emulsion would be cleared in a year's time on the shelf provided that no change in chemical constitution occurred in that interval.

4. Emulsion clearing rates essentially may measure the flotation of the population of smallest particles, which exist in both "good" and "bad" emulsions, and may not permit differentiation between two such preparations.

5. Highly unstable particles that are readily spun out of the "cream" on ultracentrifugation exist in "bad" emulsion but not in "good" emulsion. This initial spin-out of a continuous oil phase for "bad" emulsion decays with time and only accounts for a fraction of the total. The residual follows the same pattern as the spin-out of continuous oil phase in "good" emulsion.

6. "Good" emulsion demonstrates an induction period at high r.p.m. values before a continuous oil phase can be observed and may indicate a "yield" value. Subsequently, the rate of coalescence accelerates and the acceleration rate is a function of the r.p.m. and may be considered a measure of the toughness of particle "skin."

7. A consistent model for these phenomena is the more ready flotation of large oil particles so that the "cream" is a grading of large to small oil particles from top to bottom. Unstable large particles immediately coalesce as a function of their number. Stable particles coalesce to form larger ones. The larger they are, the more readily are they deformed under centrifugal stress. However, a critical number of such large ones is necessary before coalescence and the spin-out of oil in "good" emulsion.

APPENDIX

The force, F, on a particle in a gravitational field is

$$F = V(\rho - \rho')a \qquad \text{(Eq. 1)}$$

where V = volume of particle, ρ and ρ' are the densities of the particle and medium, respectively, and a is the acceleration due to the field (7). When steady state conditions are reached, there is a constant particle velocity, v, and the gravitational force equals that applied to the particle by Stokes law so that

$$V(\rho - \rho')a = k\eta v \qquad \text{(Eq. 2)}$$

where k is a shape constant and η is the coefficient of viscosity of the fluid medium (7).

Equation 2 may be solved for velocity, v, and in a centrifuge of a given r.p.m. where $2\pi x$ is the distance traveled by a particle in one rotation at a distance, x, from the axis of rotation where the acceleration is $a = u^2/x$ and u = r.p.m. $2\pi x$ is the velocity of the particle, then

$$v = dx/dt = V(\rho - \rho')\,4\pi^2\,(\text{r.p.m.})^2\,x/k\eta \qquad \text{(Eq. 3a)}$$

$$= K4\pi^2(\text{r.p.m.})^2x \qquad \text{(Eq. 3b)}$$

$$= S(\text{r.p.m.})^2x \qquad \text{(Eq. 3c)}$$

where t is in minutes.

If the initial distance of the particle is x_1 from the axis of rotation at $t_1 = 0$, and the distance at time $t_2 = t_c$ is x_2, then Eq. 3a, 3b, and 3c may be integrated between these limits and

$$\ln x_2/x_1 = S(\text{r.p.m.})^2t_c \qquad \text{(Eq. 4a)}$$

$$= K4\pi^2(\text{r.p.m.})^2t_c \qquad \text{(Eq. 4b)}$$

Under gravity, $a = g$ in Eq. 2 and

$$v = dx/dt = V(\rho - \rho')g/k\eta \qquad \text{(Eq. 5a)}$$

$$= Kg \qquad \text{(Eq. 5b)}$$

which integrates between the limits $t_1 = 0$ and $t_2 = t_g$ for x_1 and x_2 to

$$x_2 - x_1 = Kgt_g \qquad \text{(Eq. 6)}$$

The ratio of the time necessary for the particle to fall a distance $l = x_2 - x_1$ under true gravity to the time under ultracentrifugation where d is the original distance of the particle from the axis of rotation of the ultracentrifuge can be derived from Eqs. 4 and 6 and is

$$t_g/t_c = 4\pi^2\,(\text{r.p.m.})^2/g\,\ln\,(l/d + 1) \qquad \text{(Eq. 7)}$$

Since g = 980 cm./sec.^{2} = 3.53 × 10^6 cm./min.^{2}, Eq. 7 may be converted to an expression in logarithms to the base ten

$$t_g/t_c = 4.856 \times 10^{-6}l(\text{r.p.m.})^2/\log(l/d + 1) \qquad \text{(Eq. 8)}$$

This relation is derived for sedimentation; for flotation the sign is reversed.

For the set of flotation studies plotted in Fig. 17 the time interval, t_c, between the ordinate values of 0.8500 (log 7.08 cm.) and 0.8000 (log 6.31 cm.) corresponds to the flotation of a particle of l = 0.77 cm. with a d = 7.08 cm. initial distance from the axis of rotation of the ultracentrifuge. Based on r.p.m. values ranging from 12,590 to 35,600 and the respective t_c values the time, t_g, for the emulsion clearing of 0.77 cm. of emulsion[1] on the shelf under gravity alone as calculated from Eq. 8 averages 311 days. The range of calculation of t_g was 271 to 376 days.

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Spectrophotometric Study of Aqueous Solutions of Warfarin Sodium

By C. F. HISKEY, E. BULLOCK, and G. WHITMAN

The pKa of the enolic form of warfarin has been determined by a spectrophotometric technique and found to be 5.05 ± 0.1. It was found that if its solutions were prepared with an alkaline buffer they could be kept stable and free of precipitation for several weeks when stored at refrigerator temperatures.

SINCE the discovery by Link (1) of the hypoprothrombinemic properties of warfarin sodium,[1] the use of this agent both in a parenteral as well as in the oral form, has become widespread. The oral method of administration is the preferred one, but there are occasions when this route is contraindicated. This occurs, for example, in instances where the patient with a thromboembolic episode cannot swallow because of vomiting or of being comatose. In such cases it is necessary to administer the drug intravenously or intramuscularly. There is usually an initial larger dose of 25 to 50 mg., which is subsequently followed by 5 or 10-mg. daily maintenance doses.

The warfarin sodium used in the parenteral preparation is supplied in the form of a lyophilized powder, containing with it sufficient sodium chloride. When an appropriate amount of sterile water is introduced into the vial a clear isotonic solution containing about 25 mg. of the drug per ml. is obtained. It became of interest to determine whether the lyophilized warfarin powder could be reconstituted to give stable solutions, which on the succeeding days following the start of therapy, could be used for the maintenance doses.

While investigating this problem some difficulties were experienced with such solutions. Precipitates were occasionally found and, consequently, it was decided to make a systematic study of the absorption spectra of warfarin sodium solutions in order to determine the acid ionization constant of the enol form. Because of the very low solubility of the enol form, the more common potentiometric method could not be applied. The data obtained were useful in setting up the conditions for producing stable parenteral solutions and, being of general interest, are therefore presented here.

EXPERIMENTAL

Effect of pH on the Absorption Spectra of Warfarin Solutions

Warfarin powder corresponding in its properties to those described in U.S.P. XVI was used in all of these studies. Two solutions were prepared at a concentration of 6.06×10^{-5} M with respect to warfarin. One was made 0.01 N with hydrochloric acid and the other was made 0.01 N with sodium hydroxide. The absorption spectra of these two solutions were determined using a DK-2 recording spectrophotometer with 1-cm. silica cells and using water as the reference solvent.

The spectra so obtained are presented in Fig. 1 where A represents that of the enolic form and B that of the enolate form. It will be observed that the enolic form has absorption peaks at 274, 282, and 305 mμ. In addition, a peak in the vicinity of 319 mμ emerges as a shoulder on the 305 mμ peak. The enolate spectrum shows a main peak at 308 mμ with a shoulder in the vicinity of 293 mμ. There is, as would be expected, a general shifting of this spectrum toward the visible. Isosbestic points are observed at 289 and 251 mμ. A summary of the molar absorptivities is given in Table I.

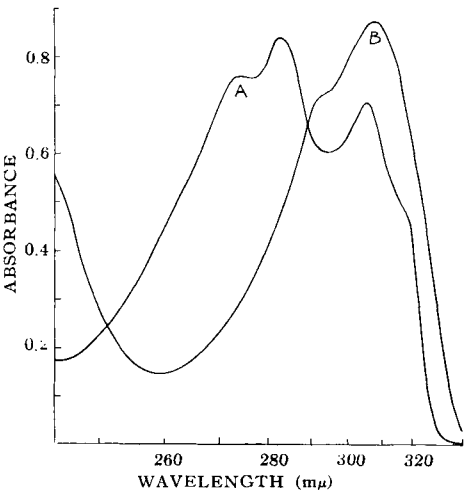


Figure 1

Received January 26, 1961, from Endo Laboratories Inc., Richmond Hill 18, N. Y.
Accepted for publication March 1, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
The authors would like to acknowledge the assistance of Dr. Nathan Weiner who suggested that this study be performed.

[1] Warfarin is the coined generic name for the synthetic hypoprothrombinemia-inducing agent 3-(alpha-acetonyl benzyl)-4-hydroxycoumarin. Coumadin sodium is the registered trademark of Endo Laboratories for this chemical.

TABLE I.—SUMMARY OF MOLAR ABSORPTIVITIES

Form	mμ	Molar Absorptivity
Enol	274	12,500
	282	13,900
	305	11,600
Enolate	308	14,400
	293	11,900

In order to determine the acid ionization constant of this compound, a group of solutions was prepared with identical molar concentrations of the anticoagulant, i.e., 5.5×10^{-5} *M*, but with a pH varying between 2 and 10. These were prepared by adding disodium hydrogen phosphate in an amount sufficient to give a 0.1 *M* solution. The pH was adjusted in a preliminary way to the desired value by adding either hydrochloric acid or sodium hydroxide, after which the solutions were brought to volume. The spectrum was then recorded for each solution, after which a final pH measurement was made. Some of the spectral curves that were obtained are shown in Fig. 2.

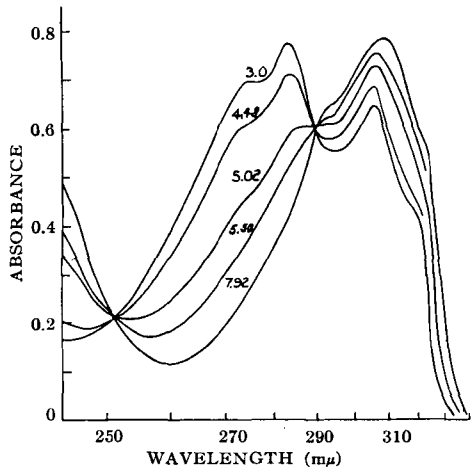


Figure 2

The data were solved for the pKa by assuming an enol-enolate equilibrium given by the simple proton transfer reaction shown in Eq. 1. From the Nernst relation we may write Eq. 2

$$\text{Enol} + H_2O \rightleftharpoons \text{Enolate}^- + H_3O^+ \quad \text{(Eq. 1)}$$

$$\text{pH} = \text{pKa} + \log \frac{(\text{Enolate})}{(\text{Enol})} \quad \text{(Eq. 2)}$$

where the terms in the brackets represent the concentration of the acid and base forms. Inasmuch as the spectrophotometric method involves a concentration determination rather than an activity determination, the expression in Eq. 2 is only approximate.

By examining the curves obtained in Fig. 2, it will be observed that the largest spectral change occurs in the 274 mμ region. This was the region chosen for our calculations since it would give us the greatest change per pH unit. The concentrations of the enolate and enol forms were found by fitting the data into an equation of the form shown in Eq. 3

$$A = a_1bc \frac{h}{h + \text{Ka}} + a_2bc \frac{\text{Ka}}{h + \text{Ka}} \quad \text{(Eq. 3)}$$

where A, is the absorbance of the solution at 274 mμ, a_1 and a_2 are the absorptivities of the enol and enolate species, respectively, b is the path length of the absorption cell, c is the concentration of the anticoagulant in the solution, and h is the activity of the hydrogen ion in these solutions. Having determined these concentration values it can be seen that substituting them into Eq. 2, a linear plot of the data should be obtained with a slope of unity. In Fig. 3 this has been done and reasonable agreement with Eq. 2 is observed. The pKa determined graphically is 5.05 ± 0.1. Thus, it is seen that the enol is an acid nearly as strong as acetic acid. At a pH of 8 or higher the warfarin will be completely in the enolate form. Consequently, clear stable solutions may be anticipated at that pH.

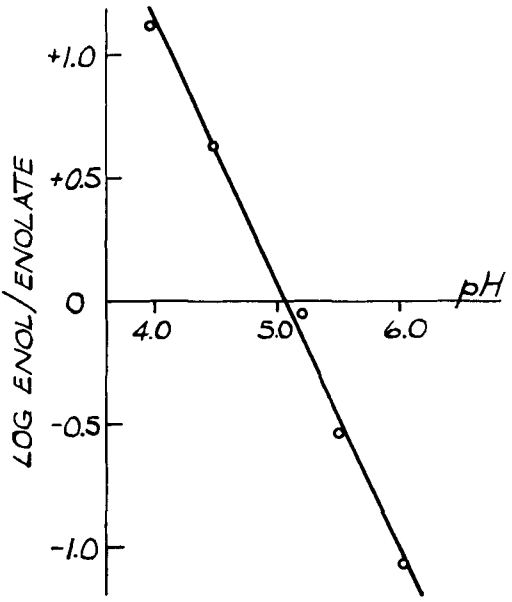


Figure 3

This study was performed in order to determine whether reconstituted vials of lyophilized warfarin sodium injection are stable enough to be used over a period of time as a multidose vial. Samples from three lots of 50-mg. vials and three lots of 75-mg. vials were reconstituted with 2 and 3 ml., respectively, of sterile water. In another study similar vials were reconstituted with similar amounts of disodium hydrogen phosphate buffer at a concentration of 10 Gm. per L. Daily withdrawals were made which were assayed for the warfarin sodium content. The vials were examined each day for the appearance of crystals and for discoloration of the solution. The reconstitution and withdrawals were made under simulated clinical conditions and the vials were stored in a refrigerator between withdrawals.

Before reconstitution, the metal caps were removed and the vials were weighed with the rubber caps and seals in place. The sterile water and disodium hydrogen phosphate solution were injected into each vial with a 5-ml. syringe and the vials were weighed to determine the precise amount of solution that had been added. The buffer solution had pre-

TABLE II.—RESULTS OBTAINED WITH A PHOSPHATE BUFFER

Daily Withdrawal Schedule, 0.2 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	23.1	23.5	23.1	23.3	22.6	23.57
Days after reconstitution						
0	23.1	23.3	23.2	23.9	22.99	23.2
1	23.5	23.7	23.4	23.9	23.2	22.9
2	23.6	23.5	23.3	23.6	23.0	22.9
3	23.9	23.8	23.4	23.9	22.7	23.0
4	23.9	23.7	23.4	23.6	23.2	22.9
7	23.6	23.6	23.45	23.9	22.5	22.8
8	23.8	23.9	23.6	24.0	23.1	22.4
9	23.8	23.8	22.8	24.2	23.2	23.1
10	23.6	23.8	23.4	...	22.9	23.1
11	23.6	23.7	23.4	...	...	...
14	23.8	23.4	23.2	...	...	...
28	23.8	23.7	23.3	...	...	...
35	...	23.8	23.5	...	...	...

There was no discoloration of solution or formation of precipitate.

Daily Withdrawal Schedule, 0.3 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	24.5	24.2	22.05	24.45	27.3	24.9
Days after reconstitution						
0	25.6	24.6	22.5	25.0	...	24.6
1	...	24.0	22.4	25.1	28.0	24.3
2	25.0	24.3	22.0	...	28.3	25.0
6	25.2	24.5	22.5	25.0	28.3	24.2
7	25.2	24.3	22.4	25.2	27.8	24.2
8	24.7	24.0	22.2	25.1	...	24.4
9	25.0	24.1	22.3	...	...	...
12	24.9	24.1	22.4	...	...	...
26	25.2	24.1	22.5	...	...	...
33	...	...	22.5	...	...	...

There was no discoloration of solution or formation of precipitate.

Daily Withdrawal Schedule, 0.4 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	24.05	23.4	23.2	23.4	23.9	23.2
Days after reconstitution						
18	...	23.8	23.4	23.9	24.8	23.8
25	24.4	23.4	23.6	24.0	24.9	23.8
26	24.8	23.7	23.5	24.1	24.6	23.6
27	24.7	23.8	23.6	24.2	...	23.8
29	24.6	23.8	23.6	24.0	24.2	23.7
32	24.3	23.6	23.7	...	...	...
33	24.5	23.5	23.6	...	...	...

There was no discoloration of solution or formation of precipitate.

viously been placed in 10-ml. bottles, sealed with rubber caps and metal seals, and autoclaved at 248° F. and 15 p.s.i. for 15 minutes.

The anticipated concentration could be computed by using the original assay value for each lot. A 1-ml. tuberculin syringe was used to remove a 0.2-ml. portion of solution immediately after reconstitution, and daily thereafter. The amount of solution withdrawn was determined by injecting the solution into small, tared, stoppered test tubes which were weighed and transferred quantitatively to a suitable volumetric flask. Dilutions were made with freshly prepared 0.01 *N* sodium hydroxide to give a concentration of warfarin sodium equal to 1 mg./100 ml.

The absorbance was determined on the Beckman DU spectrophotometer at 308 mμ, using 0.01 *N* sodium hydroxide in the reference cell. Computation of concentration was as follows:

$$\text{mg./ml.} = \frac{11.62 \times A_{308} \times W_2}{\text{ml. solution} \times W_1}$$

where W_1 = weight of sample, W_2 = total weight of container contents after addition of phosphate solution, W_1/W_2 is the fraction of the total contents of the vial taken for assay, and ml. solution refers to the volume of phosphate solution added to the vial.

Daily withdrawals of 0.3 and 0.4 ml. from other vials of these lots were made employing similar methods. Sterile conditions were maintained. All syringes were thoroughly washed, wrapped in brown paper, autoclaved at 248° F. and 15 p.s.i. for 15 minutes, and dried in an oven at 105° C. Before

each entry into a vial, the caps were rubbed with alcohol.

The results obtained with the phosphate buffer are presented in Table II. With sterile water, similar results were obtained but it was noticed after a few days that about half of the vials showed precipitation of the enol form. When precipitates were observed the vials were discarded. Since this would be unsatisfactory from a manufacturers as well as a clinical viewpoint, use of sterile water for reconstitution and multiwithdrawals is not recommended.

The data in Table II indicate that a vial of reconstituted warfarin sodium solution with disodium hydrogen phosphate buffer is quite stable over a reasonable period of time and can be used as a multidose vial.

REFERENCE

(1) Link, K. P., *Circulation*, **19**, 97(1959).

Oral *versus* Subcutaneous Potency of Codeine, Morphine, Levorphan, and Anileridine as Measured by Rabbit Toothpulp Changes

By FLOYD E. LEADERS and H. H. KEASLING†

The ratio of oral *vs.* subcutaneous potency of codeine, morphine, levorphan, and anileridine has been obtained using rabbit toothpulp threshold changes. Values for codeine, morphine, and levorphan were approximately equal. The value for anileridine was approximately one-half that of the other agents.

MORPHINE is generally recognized to be inactive when administered orally (1). Likewise, oral codeine in doses up to 60 mg. is ineffective as an analgesic agent (2, 3). Levorphan (4) and anileridine (6), however, have been reported to have approximately equivalent analgesic potency by either oral or subcutaneous routes of administration. The differences in reported efficacy of these compounds by these routes prompted this study. The change in toothpulp thresholds in the rabbit was chosen to compare the oral *vs.* subcutaneous potency of codeine, morphine, levorphan, and anileridine.

METHODS

Toothpulp threshold changes were measured as reported by Yim, *et al.* (5), using 0.7 to 1.5 Kg. rabbits. Fresh drug solutions were prepared daily in 0.9% saline and administered to the rabbit subcutaneously or orally by stomach tube.

The drugs utilized in this study were codeine phosphate, morphine sulfate, levorphan tartrate, and anileridine dihydrochloride. All doses were given in terms of μm./Kg. of the base.

The values for total area under the time-response curve were approximated as described by Winter and Flataker (7). These figures were obtained from the percentage changes of each individual rabbit. The peak percentage change during the first 90 minutes post drug was utilized in the analysis of peak responses.

A two by two parallel line bioassay was performed on all data except that of levorphan. A two by three parallel line bioassay was used in this instance. Both peak effect and total area under the curve responses were statistically analyzed by the method of Finney (8), using log response and log dose as effect and dose metameters, respectively.

RESULTS

The results of the bioassay of oral *vs.* subcutaneous doses of codeine phosphate, morphine sulfate, levorphan tartrate, and anileridine dihydrochloride are presented in Figs. 1 and 2. Log dose was plotted against both the log of the total area under the response curve and the log of the peak response. The relative potencies with fiducial limits are summarized in Table I.

Table II illustrates the results of the analysis of variance for each of these groups.

DISCUSSION

Our data demonstrate that codeine, levorphan, and morphine have subcutaneous-oral relative potency values of approximately equivalent magnitudes as measured by elevation of toothpulp thresholds in rabbits. This would be expected since they are close chemical congeners. Absolute potency, however, did vary from drug to drug. These data would thus be in agreement with the literature which

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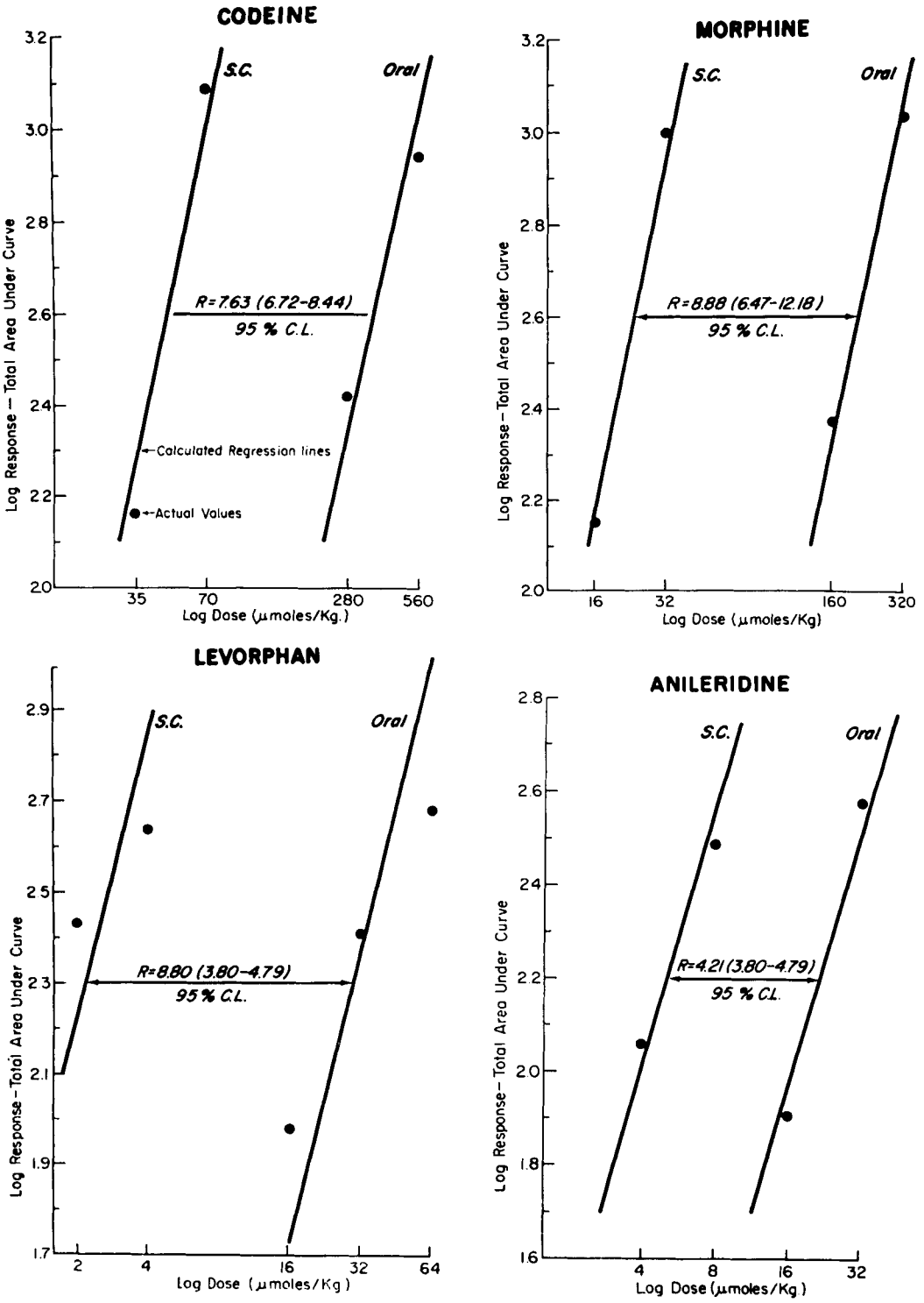


Fig. 1.—Results of bioassays of oral *vs.* subcutaneous codeine, morphine, levorphan, and anileridine. Response = total area under the curve.

reports codeine, morphine, and levorphan to be less active orally than parenterally in man. Anileridine has a relative potency value of approximately one-half that of the other compounds tested, although this figure does fall within the confidence limits for levorphan. This relative potency of anileridine is

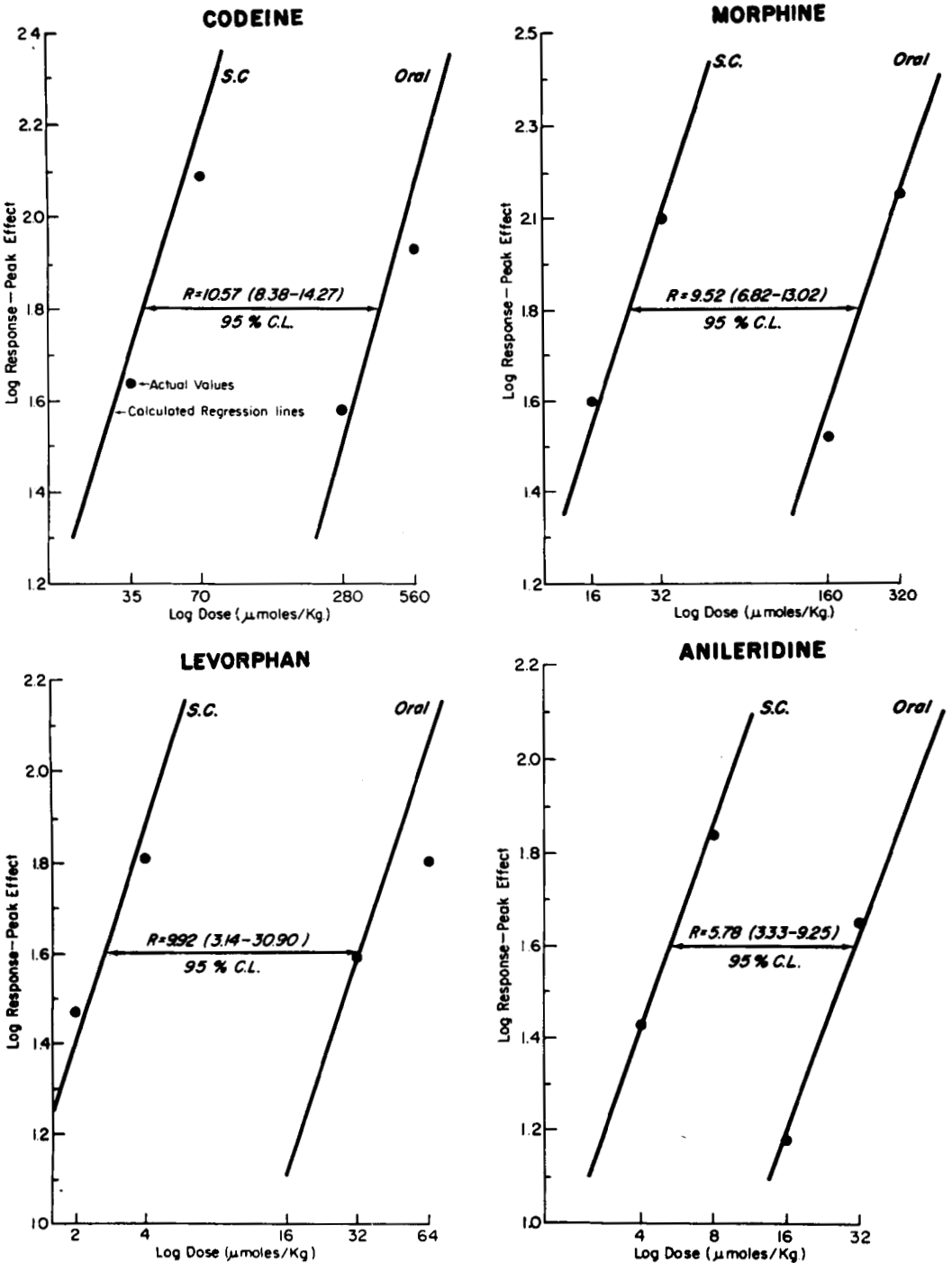


Fig. 2.—Results of bioassay of oral vs. subcutaneous codeine, morphine, levorphan, and anileridine. Response = peak effect.

similar to findings reported previously in rats (6), and would possibly be expected to differ from the values of the other agents since it is not closely related to them chemically.

Greater variations were found for peak response than for total area. Our results demonstrate that the relative potency values are greater when calculated from the peak effect figures than when calculated from the total area response data. This difference reflects the relatively lower peak effects following oral administration.

In the absence of clinical bioassay data it is

TABLE I.—RELATIVE POTENCY OF ORAL *VS.* SUBCUTANEOUS DRUG ADMINISTRATION

Drug	Total Area Under the Curve	Peak Effects
Codeine	R = 7.63 (6.72- 8.44)[a]	R = 10.57 (8.38-14.27)
Morphine	R = 8.88 (6.47-12.18)	R = 9.52 (6.82-13.02)
Levorphan	R = 8.80 (2.58-28.47)	R = 9.92 (3.14-30.09)
Anileridine	R = 4.21 (3.80- 4.79)	R = 5.78 (3.33- 9.26)

[a] Fiducial limits at the 95% confidence level.

TABLE II.—ANALYSIS OF VARIANCE OF BIOASSAY RESULTS PRESENTED IN FIGS. 1 AND 2.

Total Area Under the Curve				Peak Effects			
Source of Variation	d.f.	Mean Squares	F Ratio	Source of Variation	d.f.	Mean Squares	F Ratio
			Oral *vs.* s.c. Codeine				
Preparations	1	0.02	0.07	Preparations	1	0.42	6.63
Regression	1	5.28	19.48	Regression	1	2.47	37.42
Parallelism	1	0.42	1.55	Parallelism	1	0.21	3.18
Between doses	3	1.91		Between Doses	3	1.03	
Error	36	0.27		Error	36	0.07	
Total	39			Total	39		
			Oral *vs.* s.c. Morphine				
Preparations	1	0.16	0.40	Preparations	1	0.01	0.07
Regression	1	5.70	14.25	Regression	1	3.16	23.37
Parallelism	1	0.08	0.20	Parallelism	1	0.04	0.30
Between doses	3	1.98		Between Doses	3	1.07	
Error	36	0.40		Error	36	0.14	
Total	39			Total	39		
			Oral *vs.* s.c. Levorphan				
Preparations	1	0.39	1.22	Preparations	1	0.04	0.25
Regression	1	2.60	8.13	Regression	1	1.43	8.77
Parallelism	1	0.07	0.22	Parallelism	1	0.09	0.55
Between doses	4	1.82		Between Doses	4	0.76	
Error	45	0.32		Error	45	0.16	
Total	49			Total	49		
			Oral *vs.* s.c. Anileridine				
Preparations	1	0.01	0.04	Preparations	1	0.43	12.01
Regression	1	3.03	11.65	Regression	1	1.90	53.07
Parallelism	1	0.16	0.62	Parallelism	1	0.01	0.63
Between doses	3	1.06		Between Doses	3	0.78	
Error	36	0.26		Error	36	0.36	
Total	39			Total	39		

difficult to assess the predictive value of this procedure. It is to be hoped that the pertinent human data will be available in the near future.

SUMMARY

Changes in toothpulp threshold in rabbits have been measured following oral and subcutaneous codeine, morphine, levorphan, and anileridine. Codeine, morphine, and levorphan had approximately equal relative potency values. The relative potency value for anileridine, a structurally dissimilar compound, was approximately one-half that of the other agents. These data would thus agree with clinical reports that codeine, morphine, and levorphan are less active orally than when given parenterally. Relative potency values were greater when calculated from peak effect data than when calculated from the total area under the curves.

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Solubilization of Benzoic Acid Derivatives by Polyoxyethylene Stearates

By FRANK W. GOODHART† and ALFRED N. MARTIN

A series of five hydrophilic surfactants, all polyoxyethylene stearates,[1] was used in aqueous solution to solubilize a group of solid benzoic acid derivatives. The concentration of solubilizate rendered soluble was a linear function of surfactant concentration which is characteristic of solubilization of semipolar compounds. The critical micelle concentration of Myrj 59 was determined by two methods, and these data support the theory of micellar solubilization by nonionic surfactants. With a few exceptions, solubility was greater in the least hydrophilic surfactant solutions.

In recent years it has become quite common to employ nonionic surfactants as solubilizing agents for certain drugs. Although basic understanding regarding these phenomena is lacking, several reports have appeared regarding this type of solubilization. A nonalcoholic phenobarbital elixir has been prepared by using 2 to 5 per cent Tween 80 (1), and a parenteral solution of reserpine was stabilized using the same solubilizing agent (2). Numerous sources have pointed out the usefulness of nonionic surfactants for solubilizing vitamin A palmitate, vitamin A alcohol, vitamin D (3, 4), and volatile oils such as peppermint (5).

Correlation between the hydrophilicity of the nonionic surfactants and the chemical structure of solubilizates has not been investigated to any great extent. Cohen, using polyoxyethylene ethers of alkyl alcohols (6) as illustrated

$$C_nH_{(2n+1)}O(CH_2CH_2O)_{n'}H$$

found that if n was held constant, polar solubilizates dissolved better as n' increased (7). For a fixed n', the solubility of hydrophobic substances increased as n increased (8, 9).

Moore and Bell (10) solubilized various perfumery compounds in a series of surfactants containing 10–60 units of ethylene oxide. They found the optimum amount of ethylene oxide content for maximum solubilization differed for the various solubilizates such as benzyl alcohol, phenylethyl alcohol, benzaldehyde, and others.

It can be seen from these studies that an optimum hydrophilic-lipophilic balance exists for solubilized compounds just as it does for emulsifiable oils. Therefore, this investigation was undertaken to relate the degree of solubilization of closely related solid solubilizates to the hydrophile-lipophile character of the surfactant. A group of five polyoxyethylene stearates was chosen as the solubilizing agents. Since these surfactants were all of one chemical type differing only in ethylene oxide content, they served as an excellent series for comparing solubilization of related compounds as a function of hydrophile-lipophile balance.

EXPERIMENTAL

Materials and Reagents.—A series of five polyoxyethylene stearates (Myrj surfactants) listed in Table I; deionized-distilled water; hydrochloric acid, reagent grade; recrystallized benzoic, salicylic, *p*-aminobenzoic, and *p*-hydroxybenzoic acids; and recrystallized ethyl *p*-aminobenzoate, butyl *p*-aminobenzoate, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, and butyl *p*-hydroxybenzoate.

Table I.—Series of Polyoxyethylene Stearates Used in the Solubilization Studies

Surfactant	HLB
Myrj 49	15.0
Myrj 51	16.0
Myrj 52[a]	16.9
Myrj 53	17.9
Myrj 59	18.8

[a] Official in U. S. P. XVI as polyoxyl 40 stearate.

Instruments.—Beckman D U spectrophotometer, Cenco-du Nouy tensiometer, and a constant temperature shaking apparatus.

Solubilization Studies.—Since the presence of salts decreases the critical micelle concentration (cmc) (11) and, therefore, affects solubilization, the ionization of all solubilizates was suppressed by using hydrochloric acid solution, 0.005 *N*. This concentration of hydrochloric acid was found by experimentation over a range of concentration to be adequate. This acidic solution was used to prepare stock solutions of the Myrj surfactants and as a diluting agent for all spectrophotometric determinations.

The equilibration containers were one-half ounce screw-cap bottles. About 200 mg. of benzoic acid derivative was added to each of a series of bottles so as to provide an excess of solubilizate in all samples. Various concentrations of Myrj stock solution and hydrochloric acid solution were pipetted into each bottle so that a range of surfactant concentrations was obtained. A blank containing

Received August 15, 1960, from Purdue University, School of Pharmacy, Lafayette, Ind.
Accepted for publication March 6, 1961.
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[1] Myrj surfactants were supplied through the courtesy of Atlas Powder Co., Wilmington, Del.

only the solubilizate and the hydrochloric acid solution was also prepared for each run. The samples were capped and sealed around the necks with a strip of water-resistant plastic tape. The samples were equilibrated in a shaking apparatus at a temperature of 25.0 ± 0.1° for a period of forty-six to fifty hours. This time was shown by preliminary experiments to be sufficient for equilibration. After equilibrium had been reached, aliquots were taken by means of a pipet fitted with a cotton filter. Sufficient dilutions were made so that the solutions could be read spectrophotometrically at the wavelength of maximum absorption for each solubilizate. In all instances, the dilutions were large enough so that no absorption resulted from the presence of the Myrj compounds.

Determination of the cmc of Myrj 59.—Above a concentration of surface-active agent known as the critical micelle concentration (cmc), surfactant molecules form colloidal aggregates known as micelles. Two methods were employed to determine the cmc of Myrj 59 solutions. A solubility study was carried out in dilute Myrj 59 solutions. The method employed was the same as the solubility study previously discussed. A break in the solubility curve is indicative of micelle formation (12).

In order to corroborate the cmc determined by solubility equilibria, surface tensions of dilute Myrj 59 solutions were measured. The Cenco-du Nouy tensiometer was calibrated according to the conventional method (13), and the correction factor of Harkins and Jordan (14) was used to correct the observed readings. The cmc for Myrj 59 was found more precisely by saturating the surfactant solutions with benzoic acid. The sharp break occurring in the surface tension-concentration curve is again indicative of the cmc (12).

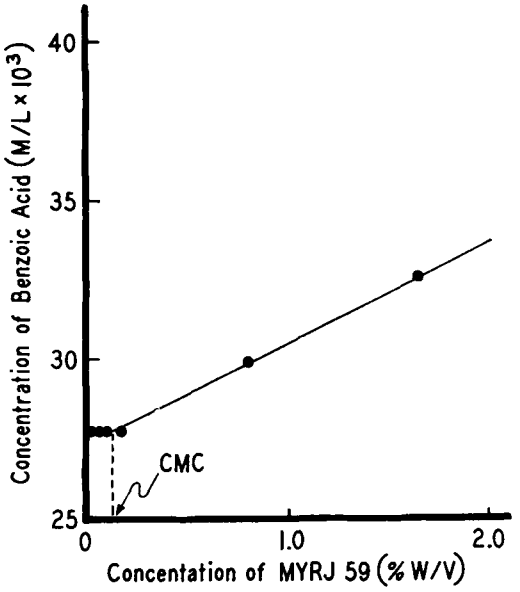


Fig. 1.—Solubility of benzoic acid at 25° illustrating one method of obtaining the critical micelle concentration (cmc).

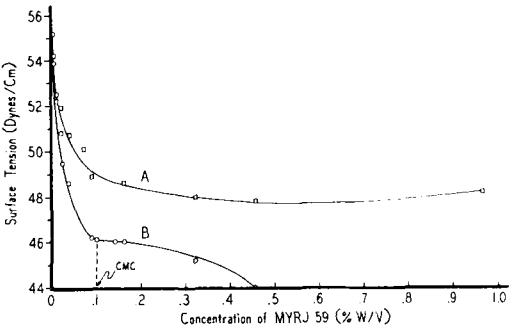


Fig. 2.—Surface tensions of Myrj 59 solutions at 23° illustrating cmc. A, In 0.005 *N* hydrochloric acid; *B*, in 0.005 *N* hydrochloric acid saturated with benzoic acid.

RESULTS AND DISCUSSION

Critical Micelle Concentration.—Figure 1 illustrates the solubility of benzoic acid in dilute Myrj 59 solutions. Up to a Myrj 59 concentration of about 0.1%, the solubility of benzoic acid is not increased. Above this concentration, benzoic acid is solubilized, presumably by the micellar aggregates of surfactant which form.

The surface tensions of Myrj 59 solutions are illustrated in Fig. 2*A*. The reduction in surface tension indicates the surfactant molecules are aggregating and forming micelles (12) until a practically constant limiting value is obtained. No cmc can be deduced from this curve. The curve representing the surface tensions of Myrj 59 saturated with benzoic acid (Fig. 2*B*), however, shows a sharp break at a surfactant concentration of 0.1%. The significance of this break is that the cmc has been reached and the solutions acquire new physical properties making them capable of solubilizing insoluble compounds (12). The agreement of a cmc of about 0.1% for Myrj 59 by both the solubility and surface tension measurements supports this fact.

Solubilization and Complexation.—It is a well established fact that carboxylic acids and phenols undergo complexation with surfactants of the polyether class (15, 16). Moreover, the present study indicates that micellar solubilization of benzoic acid occurs in solutions of polyoxyethylene stearates above the cmc.

The colloid chemist has attributed increased solubility above the cmc to true or micellar solubilization. While Myrj surfactants do contain some free polyglycol (17), results obtained from measuring cmc indicate the principal mechanism of solubilization in this study is micellar solubilization. However, Patel and Kostenbauder (18) in their studies on the interaction of *p*-hydroxybenzoic acid esters with Tween 80 showed that a positive intercept on a Langmuir-type plot may be used to differentiate between complex formation and micellar solubilization.

Since complexation should be evident below the cmc where micellar solubilization is not a factor, and since no interaction was observed in this investigation by the solubilization method used, it is reasonable to conclude that complexation is insignificant in this study below the cmc. Whether

TABLE II.—MOLAR RATIOS OF SOLUBILIZATE TO SURFACTANT OBTAINED BY PLOTTING ($S - S_0$) AGAINST ETHYLENE OXIDE CONCENTRATIONS (EQUIV./L.)[a]

Solubilizate	Myrj 49	Myrj 51	Myrj 52	Myrj 53	Myrj 59
Benzoic acid	0.0670	0.0490	0.0440	0.0366	0.0259
p-Hydroxybenzoic acid	0.0669	0.0691	0.0655	0.0564	0.0512
o-Hydroxybenzoic acid	0.0643	0.0492	0.0397	0.0386	0.0223
p-Aminobenzoic acid	0.0484	0.0412	0.0450	0.0361	0.0264
Ethyl *p*-aminobenzoate	0.0395	0.0305	0.0277	0.0277	0.0135
Butyl *p*-aminobenzoate	0.0554	0.0386	0.0313	0.0249	0.0134
Methyl *p*-hydroxybenzoate	0.0495	0.0403	0.0359	0.0309	0.0183
Propyl *p*-hydroxybenzoate	0.0453	0.0318	0.0286	0.0228	0.0130

[a] Percentages of ethylene oxide per surfactant molecules calculated from formula of Griffin (21). The percentages are: Myrj 49, 75.0% w/w; Myrj 51, 80.0% w/w; Myrj 52, 84.5% w/w; Myrj 53, 89.5% w/w; and Myrj 59, 94.0% w/w. Concentrations were corrected for water content.

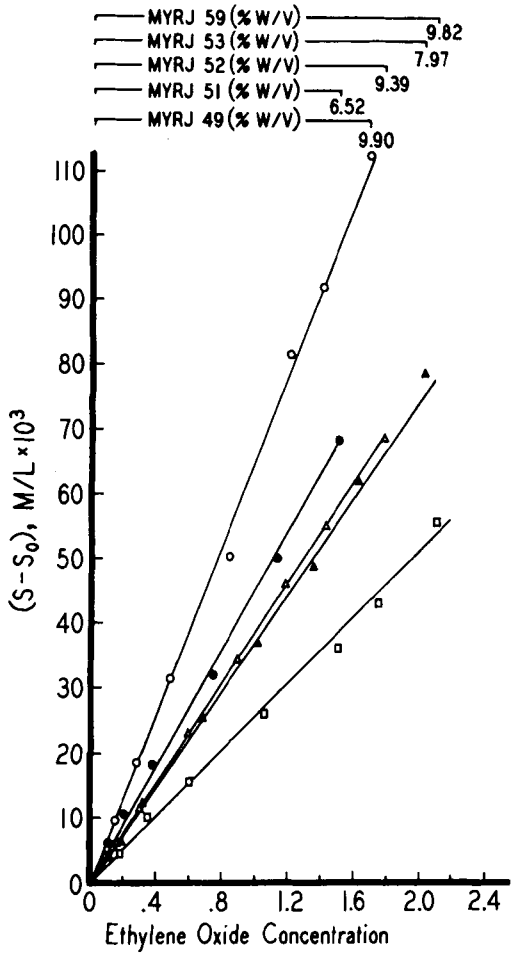


Fig. 3.—Solubilities of benzoic acid in Myrj solutions at 25°. ○, Myrj 49; ●, Myrj 51; △, Myrj 52; ▲, Myrj 53; □ Myrj 59.

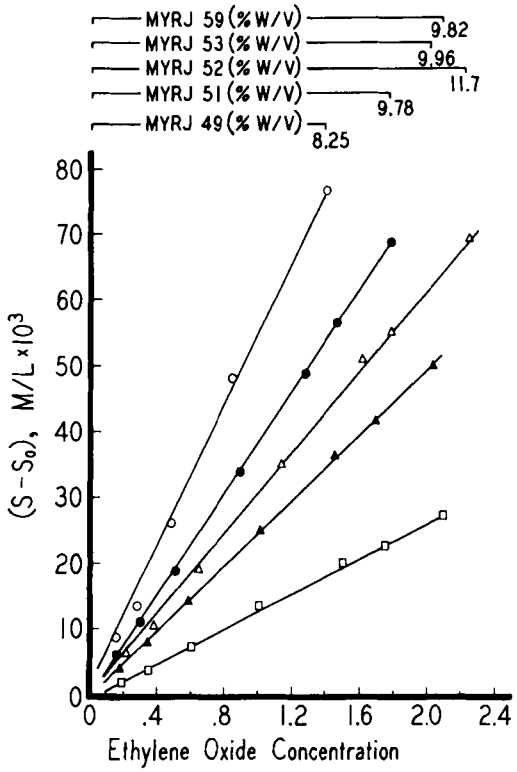


Fig. 4.—Solubilities of butyl *p*-aminobenzoate in Myrj solutions at 25°. ○, Myrj 49; ●, Myrj 51; △, Myrj 52; ▲, Myrj 53; □, Myrj 59.

or not the two effects occur together above the cmc cannot be decided by the present experimental method.

Degree of Solubilization.—The quantity of solubilizate brought into solution by the surfactant was calculated by subtracting the solubility in the acidified water solution, S_0, from the solubility, S, in the Myrj solutions. This quantity ($S - S_0$) was plotted against the surfactant concentration in equivalents of ethylene oxide per L. on rectangular coordinate graph paper. Figures 3, 4, and 5 show the solubilization of benzoic acid, butyl *p*-aminobenzoate,[2] and propylparaben by the five surfactants studied. The results are typical of the solubilization curves obtained with all solubilizates. The linear relationships obtained with each of the five Myrj compounds correspond to results obtained by other workers using nonionic surfactants to solubilize semipolar molecules (18–20).

The moles of solubilizate per equivalent of ethylene oxide in the surfactant were obtained by calculating the slopes of the solubilization isotherms and are listed in Table II. From these data the

[2] Butesin is the trade name of Abbott Laboratories, North Chicago, Ill., for butyl *p* aminobenzoate.

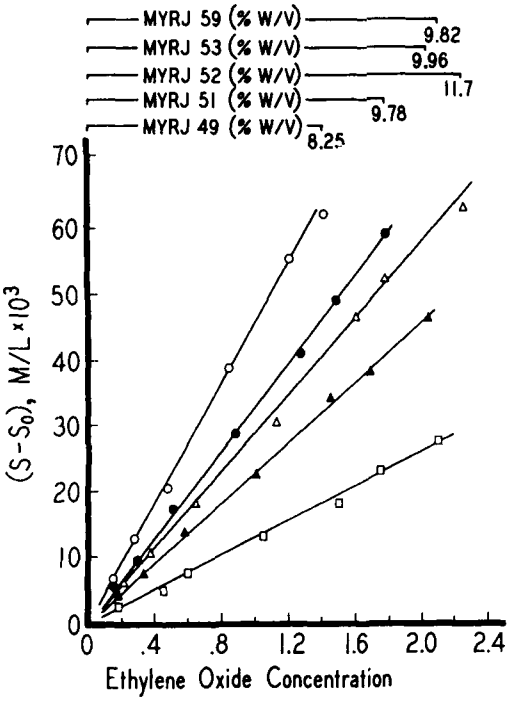


Fig. 5. —Solubilities of propylparaben in Myrj solutions at 25°. O, Myrj 49; ●, Myrj 51; △, Myrj 52; ▲, Myrj 53; □, Myrj 59.

quantity of solubilizate brought into solution by a specific concentration of Myrj 49, 51, 52, 53, or 59 can be calculated. Since the solubility in the acidified water has been subtracted, the intercepts of these curves are zero.

If the moles of solubilizate could be plotted against the moles per L. of surface-active agent, the slopes would be greatest for the most hydrophilic surfactant, i.e., the surfactant containing the longest polyoxyethylene chain. However, when molar solubilization is plotted against equivalents of ethylene oxide, the moles of solubilizate taken into the micelle per individual ethylene oxide unit is measured. The slope of such a plot shows that the efficiency of solubilization in these terms decreases with increasing length of the polyoxyethylene chain, i.e., with increasing hydrophilicity or molecular weight.

Solubilization and Chemical Structure of the Solubilizate.—The differences in the degree of solubilization for the various solubilizates can be compared on the basis of molar ratios. As shown in Table II, the molar ratios for benzoic, salicylic, and *p*-aminobenzoic acids were quite similar in all five surfactant solutions. However, *p*-hydroxybenzoic acid was solubilized to a significantly greater degree than these three acids. In general, the alkyl esters of *p*-hydroxybenzoic and *p*-aminobenzoic acids show a marked decrease in molar ratio when compared to the free acids. As a rule, all solubilizates have maximum solubilities in the least hydrophilic surfactant solutions and minimum solubilities in the most hydrophilic surfactant solutions when molar ethylene oxide concentrations are used as a criterion.

Solubilization Type.—Above the cmc, a solubilized compound is contained within the hydrocarbon layers of the surfactant or is adsorbed between the hydrophilic portions of the micelle known as the palisade layer (22). The solubilizate may also be adsorbed on the surface of the micelle (23). Nonpolar compounds are solubilized in the hydrocarbon interior of the micelle as evidenced by an increase in long X-ray spacings, while polar solubilizates are solubilized in the palisade layers of the micelle and show no change or a decrease in X-ray spacings (24). Above the cmc, the molar ratio of a nonpolar solubilizate to surfactant varies with the concentration of the solubilizing agent, while the molar ratio of a polar solubilizate is constant (22, 23). Since the solubilizates employed in this work were partially polar and since constant molar ratios were obtained (Figs. 3, 4, and 5), it is reasonable to believe the principal interaction causing increased solubility is associated with the polyoxyethylene portion of the surfactant. A possible explanation is that of Beckett, *et al.* (16), which proposes some displacement of water molecules from the oxygen atoms in the glycol chain and the resultant attraction of phenol type compounds to the oxygen atoms.

Degree of Binding of *p*-Hydroxybenzoic Acid and Its Esters in Myrj Solutions.—Figure 6 illustrates the ratio of total to free methylparaben as a function of concentration of the five Myrj compounds studied. It has been pointed out that a plot of this type can be deduced from solubility data as well as dialysis studies (18). The inhibition of anti-

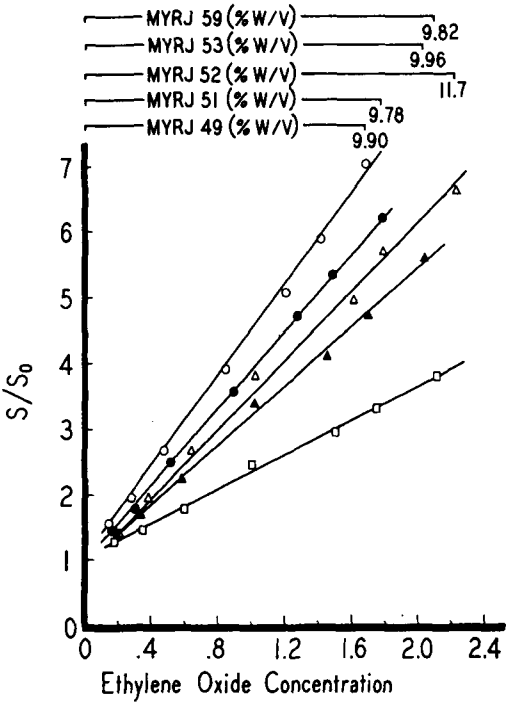


Fig. 6.—Binding of methylparaben in various Myrj solutions at 25° illustrating the ratio of bound to free paraben as a function of Myrj concentration. O, Myrj 49; ●, Myrj 51; △, Myrj 52; ▲, Myrj 53; □, Myrj 59.

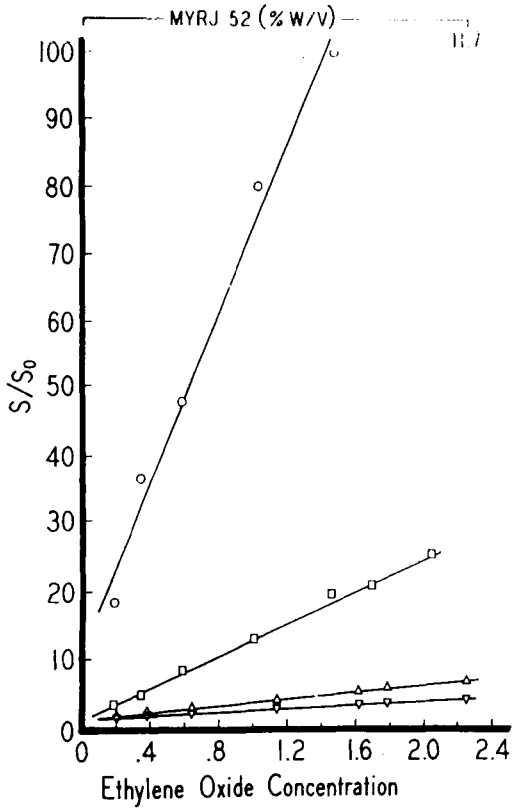


Fig. 7.—Binding of *p*-hydroxybenzoic acid and three of its alkyl esters in Myrj 52 solution at 25° illustrating the ratio of bound to free solubilizate as a function of Myrj 52 concentration. O, Butylparaben; □, propylparaben; △, methylparaben; ▽, *p*-hydroxybenzoic acid.

bacterial action by esters of *p*-hydroxybenzoic acid in the presence of nonionic surfactants has been well recognized by the pharmaceutical chemist (16). From graphs such as Fig. 6, the percentage of bound to free preservative can be calculated, thus giving the effective concentration of preservative employed (18, 25).

Figure 7 compares the binding of *p*-hydroxybenzoic acid and its butyl, propyl, and methyl esters in Myrj 52 solutions. It can be observed in Fig. 7 that an increase in the ester alkyl chain length enhances binding to Myrj 52. The binding is particularly pronounced for butylparaben which is 99% bound in a 7% Myrj 52 solution. Butylparaben, however, forms a milky white turbid solution in high concentrations of Myrj 52. This phenomenon is not at all uncommon to solubilization by nonionic surfactants, but was not observed in any other system employed in this study.

SUMMARY

1. The relationship between the hydrophilicity of five polyoxyethylene stearates on the solubilization of a group of solid benzoic acid derivatives has been studied.

2. In general, the solubilities of the compounds studied were greatest in the least hydrophilic surfactant.

3. The linear solubility isotherms and the value of critical micelle concentration are characteristic of solubilization of semipolar compounds by nonionic surface-active agents.

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Effect of Certain Drugs on Perfused Human Placenta I

Narcotic Analgesics, Serotonin, and Relaxin

By RONALD F. GAUTIERI and HENRY P. CIUCHTA†

Perfusion studies conducted on full term human placentas yielded results indicating that morphine sulfate exhibited a direct vasoconstrictor action on placental vessels. This effect was also observed with meperidine hydrochloride and codeine sulfate, although the action of the latter agent was comparatively weak. Nalorphine hydrochloride antagonized the constrictor effect of morphine sulfate and codeine sulfate, but not that produced by meperidine hydrochloride. In addition, serotonin and relaxin both elicited powerful transient vasoconstriction.

IT IS GENERALLY concluded that the primary circulatory action of morphine is vasodilation, which is due to relaxation of blood vessels (1). Hypotension in cats and dogs (2), and cutaneous vasodilation of the flush area in man and animals (3) caused by morphine, support this concept. The mode of action for this effect is probably mediated centrally and peripherally and not directly upon the blood vessels involved, for vascular relaxation is not in accord with the principal action of morphine on smooth muscle elsewhere in the body which usually is stimulated to contract.

Many investigators, including von Euler (4), and Eliasson and Aström (5), have reported the effects of a number of drugs on perfused placental vessels, but there is no available information in the literature concerning the action of morphine on these vessels. Since morphine has been shown to have paradoxical actions on different smooth muscle, it becomes imperative to discern the effect that it may have on placental vasculature.

Narcotic analgesics, such as morphine (6), meperidine (7), and methadon (8), are well known to cross the placental barrier without much difficulty. Since this occurs with therapeutic dosage, the risk of neonatal respiratory depression is very high. With the advent of N-allylnormorphine (9), fortunately, respiratory depression, if present in the newborn, can be alleviated readily (6). Although the respiratory depression is undoubtedly due to the effect of the narcotic on the fetal respiratory center, the possibility arises that part of the depression may be due to decreased oxygenation of fetal blood brought about by a decreased blood flow through placental vessels which have been constricted by a direct acting drug.

The human placenta has been shown to be devoid of nervous tissue by Schmitt (10) and Spivack (11). Thus, it may be used as a nerve-free preparation in which direct effects of drugs acting on the smooth muscle of the linings of its blood vessels may be clearly elucidated. Therefore, it is the main purpose of this investigation to observe the effects brought about on the placental vasculature by not only morphine but also other narcotic agents, and in the event that there are direct effects, attempt to antagonize them.

MATERIALS AND METHODS

Full term placentas were obtained from the hospital within 5–15 minutes after normal delivery. Each was transported to the laboratory in a container of preheated (38°) Tyrode's solution which was modified by the addition of 0.525% polyvinylpyrrolidone (Plasdone C).

The preparation subsequently was flushed free of blood by injecting 10% sodium citrate solution into one of the arteries. Cannulae were inserted into the vein and one artery of the umbilical cord 5–10 cm. from the placenta. The remaining umbilical artery was ligated. The whole preparation was then placed into a perfusion chamber containing the modified Tyrode's solution which was maintained at a constant 37° by means of a Bronwill thermoregulator. The arterial cannula was connected with rubber tubing to a hydrostatic reservoir which was adjusted to yield pressures ranging between 50–60 mm. Hg. The solution in the reservoir, which had a pH range of 7.2–7.4, was maintained at 37° and aerated. The venous outflow, ranging between 10–60 ml./min., was measured directly and not reperfused.

A total of six placentas were discarded for quantitative reasons, i.e., the outflow per minute was either negligible or so far below the average preparation that it was considered impractical to measure.

Received April 28, 1961, from Temple University, School of Pharmacy, Philadelphia 40, Pa.
Accepted for publication May 19, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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The authors wish to express sincere thanks to the members of the obstetrical staff of Temple University Hospital for the generous supply of placentas; to Drs. D. E. Mann, Jr., and E. J. Larson for their valuable suggestions and discussions, and to Messrs. J. C. Tatnall and P. DeLuca for their technical assistance.

In 30 successful perfusion experiments the average duration of each preparation was between 2 and 3 hours.

The following drugs were injected into the rubber tubing adjacent to the arterial cannula in a volume of distilled water not exceeding 1 ml.: morphine sulfate, meperidine hydrochloride, codeine sulfate, nalorphine hydrochloride, 5-hydroxytryptamine creatinine sulfate (serotonin), relaxin, and alcoholic amyl nitrite.

RESULTS

Morphine.—Morphine sulfate in doses of 0.2–0.4 mg. (in eight preparations) had a pronounced constrictor action on the placental vessels. This effect (Fig. 1) was sustained and characterized by a slowly progressive decrease in the volume flow rate. The reduction in the flow rate ranged between 35–92% (Table I). Upon subsequent administration of 5 mg. of nalorphine, the morphine-induced vasoconstriction was antagonized, with the flow rate returning to near normal (Fig. 2, Table II).

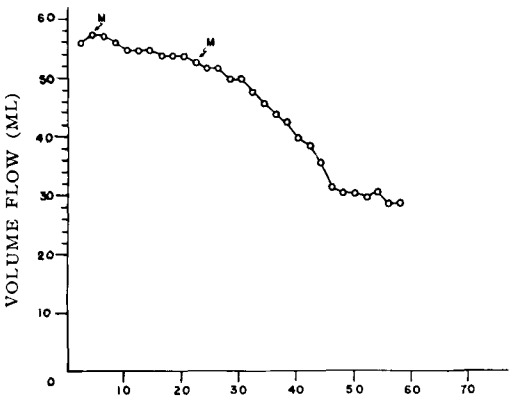


Fig. 1.—Effect of morphine sulfate on volume flow rate in vessels of the perfused human placenta. M, Morphine, 0.2 mg.

TABLE I.—COMPOSITE RESULTS

Drug	No. of Experiments	% Reduction in Flow Rate (Range)
Morphine	8	35–92
Meperidine	3	39–42
Codeine	3	6–11
Serotonin	5	19–61
Relaxin	3	59–84

TABLE II.—NALORPHINE ANTAGONISM OF NARCOTIC-INDUCED VASOCONSTRICTION

Drug	No. of Experiments	Av. % Return to Preinjection Flow Rate
Morphine-nalorphine	3	90
Codeine-nalorphine	3	91
Meperidine-nalorphine	2	0

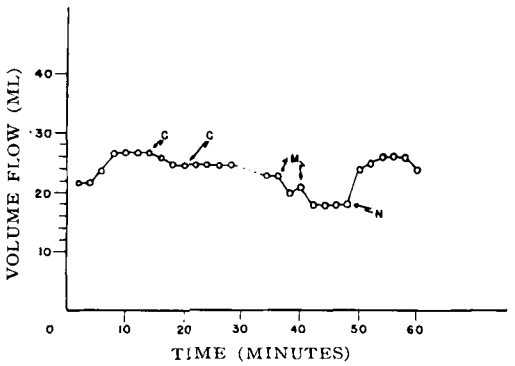


Fig. 2.—Effect of codeine sulfate and morphine sulfate on volume flow rate in vessels of the perfused human placenta and nalorphine antagonism. C, Codeine, 1.0 mg.; M, Morphine, 0.2 mg.; N, Nalorphine, 5.0 mg.

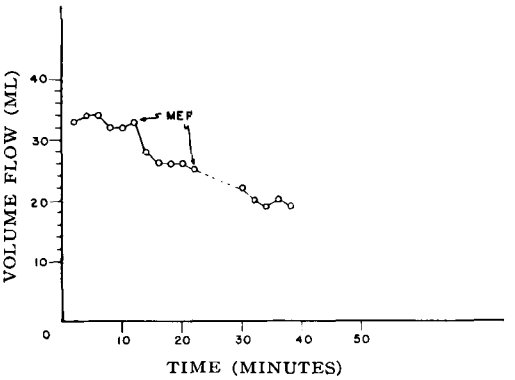


Fig. 3.—Effect of meperidine hydrochloride on volume flow rate in vessels of the perfused human placenta. MEP, Meperidine, 1.0 mg.

Meperidine.—In three experiments, meperidine hydrochloride in doses of 1–2 mg. produced a reduction in flow rate, as noted in Fig. 3, qualitatively similar to that which was observed with morphine. The per cent reduction in flow rate, which ranged between 39–42% (Table I), though not as great as that obtained with morphine, was also sustained.

Although meperidine produced a response akin to that of morphine, nalorphine was unable to antagonize it (Table II). However, the administration of 1 ml. of alcoholic amyl nitrite (1.8% v/v amyl nitrite) following nalorphine was successful in bringing about a marked increase in the flow rate.

Codeine.—Codeine sulfate in doses of 1–2 mg. (Fig. 2) produced a slight vasoconstriction in three preparations, resulting in a decrease in the flow rate of 6–11% (Table I). The administration of nalorphine antagonized this effect (Table II).

Nalorphine.—Nalorphine hydrochloride, 5–10 mg., used alone in a fresh preparation, was unable to exert any discernible effect on placental circulation. When it was administered prior to an injection of 0.2–0.4 mg. of morphine it was observed to block completely any vasoconstrictor action that this agent normally had on the placental vessels.

Serotonin.—In five experiments employing serotonin creatinine sulfate, a powerful vasoconstrictor of

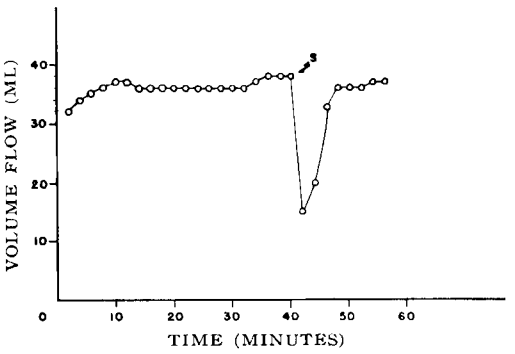


Fig. 4.—Effect of serotonin on volume flow rate in vessels of the perfused human placenta. S, Serotonin, 0.05 mg.

placental vessels, the results compared favorably to those of Astrom and Samelius (12) but the degree of constriction was not as marked. The dose of serotonin used was 50 mcg. in 0.5 ml. distilled water.

As seen in Fig. 4, after obtaining a consistent flow rate, the injection of serotonin caused an immediate decrease in this rate with almost a complete recovery to the preinjection level in approximately 6–8 minutes. The per cent reduction in the flow rate, as noted in Table I, ranged between 19–61 %.

Relaxin.—A dose of 1 ml. of relaxin[1] produced an immediate vasoconstriction in three preparations. The reduction in the flow rate produced by relaxin varied between 59–84% (Table I) during the succeeding 2 minutes. Most of this effect, although very pronounced, wore off in about 10 minutes.

DISCUSSION

The results of these experiments indicate that morphine exerts a direct constricting effect on the smooth muscle found in the linings of placental vessels. These findings are in contrast to the effects of morphine on other vascular beds where usually vasodilation is observed (2, 3). However, the constriction which occurred was analogous to that which morphine produces on gastrointestinal smooth muscle in that it was progressive and sustained.

Dornhorst, *et al.* (13), have shown that epinephrine and norepinephrine, in effective dosage, cause fetal asphyxia and cyanosis of the placenta in the rabbit and guinea pig. Because these conditions occur in response to drugs which act directly on placental vessels to constrict them, it may be concluded that any other agent which acts directly on these vessels causing vasoconstriction may also cause fetal asphyxia and placental cyanosis. Therefore, it is obvious that since morphine has been shown in this investigation to be capable of producing direct vasoconstriction in placental vessels, it may contribute partly to the fetal asphyxia sometimes caused by morphine. Of course the results reported here with morphine were those occurring *in vitro*, but it seems rather likely that similar effects would also be manifested *in vivo*.

Nalorphine was observed (Table II) to antagonize readily morphine-induced vasoconstriction in placental vessels, and it is probable that this would also occur *in vivo*. Consequently, its use *in vivo* would not only prevent central respiratory depression in the newborn but also any decreased oxygenation of fetal blood which may occur in response to the direct constricting action of morphine on these vessels.

The vasoconstrictor effect brought about by codeine in three preparations was very mild and susceptible to antagonism by nalorphine (Table II). Therefore, it would appear that fetal asphyxia due to codeine would be less marked than that produced by morphine and would be mainly due to just central respiratory depression.

Meperidine appeared to exert an effect on placental vessels that was similar to that produced by morphine. However, in the dosage employed, nalorphine was not able to antagonize the vasoconstriction caused by meperidine (Table II). Possibly if larger doses of meperidine were used, nalorphine may have exerted some antagonistic action, for it is well known that in order for nalorphine to cause withdrawal symptoms in the addict, at least 2,000 mg. must be ingested per day (14). Therefore, on this basis it seems likely that some antagonism of the constricting action of meperidine might have occurred if very large doses had been employed.

Serotonin was used in all preparations that were utilized as controls in this experiment. Five such preparations were set up and allowed to run for varying time intervals to discern any changes in the normal flow rate/min. through the placental vessels. In each case there was a period of 5–15 minutes where the flow rate increased slowly and then leveled off to a point where it would not change by more than 6% in one hour. In order to obtain effects similar to Astrom and Samelius (12), 50 mcg. of serotonin had to be administered. In each case a very profound vasoconstriction was observed which was greatest during the first 2 minutes immediately following the injection. This effect was transient, for within 4–5 minutes, the preinjection flow rate/min. was approximated. Succeeding doses of serotonin brought about a similar reduction in the flow rate as long as a sufficient time interval was allowed between injections. In a few instances, where serotonin administrations were made before the original flow rate was obtained, there occurred a diminished response to the usual vasoconstrictor effect which may be considered to be a tachyphylactic phenomenon.

Three placentas were treated with relaxin in order to observe whether this hormone of late pregnancy would cause any direct effect on placental vessels. In all cases a very pronounced constrictor action was observed. This action was transient, but the original volume flow rate/min. was never attained. This leads one to hypothesize that the constrictor effect of relaxin may be due to a contaminant in the tissue extract or even the relaxin itself. Should relaxin cause a constrictor effect *in vivo*, it may play an important role in fetal asphyxia.

The vasodilation which occurred with alcoholic amyl nitrite was probably due to the ethyl alcohol and amyl nitrite which are both known to exert a direct relaxing effect on smooth muscle. Nitrates (5) have been reported to cause a similar result on placental vessels, so these findings were anticipated.

[1] Marketed by Warner-Chilcott as Releasin.

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Comparison of the Gastrointestinal Absorption of Aluminum Acetylsalicylate and Acetylsalicylic Acid in Man

By GERHARD LEVY and BERNICE A. SAHLI

The gastrointestinal absorption rate and biological availability of aluminum acetylsalicylate have been studied in human subjects by the urinary excretion method. Acetylsalicylic acid absorption from orally administered aluminum acetylsalicylate was found to be less rapid than from aspirin, probably due to the very slow dissolution of the aluminum salt in gastrointestinal fluids. In the form used in this study, aluminum acetylsalicylate was incompletely absorbed after oral administration. Dosage forms containing this drug must be carefully evaluated with respect to absorption rate and biological availability.

THE ALUMINUM salt of acetylsalicylic acid has been admitted recently to the National Formulary (1) and is used currently in a number of proprietary pharmaceuticals. According to the background information which accompanied the proposed monograph on aluminum acetylsalicylate (Al.ASA) at the time it was considered for admission to the National Formulary (2), the drug has several significant advantages over acetylsalicylic acid (ASA). These are greater palatability, stability, and absence of astringent taste or acetic odor which is often found with ASA. As a result of its relative inertness, Al. ASA is said to be compatible with many more drugs than is ASA.

Neither the official monograph, nor the introductory statement which accompanied it at the time of submission, made reference to any possible difference in the rate or extent of gastrointestinal absorption of Al.ASA as compared with ASA. Al.ASA is a constituent of certain proprietary pharmaceuticals which are intended, among others, for the relief of acute pain and fever. Since Al.ASA is practically insoluble in water (1, 3), the possibility suggests itself that the absorption rate of the drug from the gastrointestinal tract of man may be considerably less than the absorption rate of ASA. If this is the case, there is the further possibility that Al.ASA is absorbed only partially, and that a portion of the undissolved drug is excreted in the feces. Accordingly, the gastrointestinal absorption rate and biological availability of Al.ASA have been investigated and compared with those of ASA. The present communication reports the results of this investigation and also presents certain physical-chemical data pertinent to the interpretation of the biological observations.

Received April 24, 1961, from the Biopharmaceutics Laboratory of the University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.
Accepted for publication May 19, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
The authors wish to acknowledge gratefully the cooperation of the volunteers who served as test subjects for this investigation.

EXPERIMENTAL

Materials.—Acetylsalicylic acid U.S.P., aluminum acetylsalicylate N.F. With each drug, only the fraction passing through a 100-mesh sieve was used in the investigation. Both compounds were assayed colorimetrically (4) after alkaline hydrolysis and were found to contain 100 ± 0.5% of the theoretical amount of salicylate.

Absorption Tests.—Healthy male adults served as test subjects. Their weights and ages are listed in Tables I and II. In the initial absorption test utilizing nine subjects, the drugs were administered

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Comparison of the Gastrointestinal Absorption of Aluminum Acetylsalicylate and Acetylsalicylic Acid in Man

By GERHARD LEVY and BERNICE A. SAHLI

The gastrointestinal absorption rate and biological availability of aluminum acetylsalicylate have been studied in human subjects by the urinary excretion method. Acetylsalicylic acid absorption from orally administered aluminum acetylsalicylate was found to be less rapid than from aspirin, probably due to the very slow dissolution of the aluminum salt in gastrointestinal fluids. In the form used in this study, aluminum acetylsalicylate was incompletely absorbed after oral administration. Dosage forms containing this drug must be carefully evaluated with respect to absorption rate and biological availability.

THE ALUMINUM salt of acetylsalicylic acid has been admitted recently to the National Formulary (1) and is used currently in a number of proprietary pharmaceuticals. According to the background information which accompanied the proposed monograph on aluminum acetylsalicylate (Al.ASA) at the time it was considered for admission to the National Formulary (2), the drug has several significant advantages over acetylsalicylic acid (ASA). These are greater palatability, stability, and absence of astringent taste or acetic odor which is often found with ASA. As a result of its relative inertness, Al. ASA is said to be compatible with many more drugs than is ASA.

Neither the official monograph, nor the introductory statement which accompanied it at the time of submission, made reference to any possible difference in the rate or extent of gastrointestinal absorption of Al.ASA as compared with ASA. Al.ASA is a constituent of certain proprietary pharmaceuticals which are intended, among others, for the relief of acute pain and fever. Since Al.ASA is practically insoluble in water (1, 3), the possibility suggests itself that the absorption rate of the drug from the gastrointestinal tract of man may be considerably less than the absorption rate of ASA. If this is the case, there is the further possibility that Al.ASA is absorbed only partially, and that a portion of the undissolved drug is excreted in the feces. Accordingly, the gastrointestinal absorption rate and biological availability of Al.ASA have been investigated and compared with those of ASA. The present communication reports the results of this investigation and also presents certain physical-chemical data pertinent to the interpretation of the biological observations.

Received April 24, 1961, from the Biopharmaceutics Laboratory of the University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.
Accepted for publication May 19, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
The authors wish to acknowledge gratefully the cooperation of the volunteers who served as test subjects for this investigation.

EXPERIMENTAL

Materials.—Acetylsalicylic acid U.S.P., aluminum acetylsalicylate N.F. With each drug, only the fraction passing through a 100-mesh sieve was used in the investigation. Both compounds were assayed colorimetrically (4) after alkaline hydrolysis and were found to contain 100 ± 0.5% of the theoretical amount of salicylate.

Absorption Tests.—Healthy male adults served as test subjects. Their weights and ages are listed in Tables I and II. In the initial absorption test utilizing nine subjects, the drugs were administered

TABLE I.—URINARY EXCRETION OF APPARENT SALICYLATE[a] AFTER ORAL ADMINISTRATION OF EQUIVALENT DOSES[b] OF ASPIRIN AND ALUMINUM ACETYLSALICYLATE

Subject	Age, yr.	Weight, lb.	One Hour		Two Hours	
			ASA	Al.-ASA	ASA	Al.-ASA
A	31	155	7.29	4.69	29.4	17.0
B	26	218	10.2	5.63	55.1	35.3
C	22	152	21.1	4.98	55.9	16.3
D	21	172	9.03	8.43	36.7	14.5
E	27	170	20.2	3.62	70.9	13.7
F	27	166	15.7	4.02	45.9	14.7
G	22	155	11.2	3.93	67.6	22.8
H	22	187	8.99	6.34	30.8	19.9
I	22	150	28.2	5.42	66.1	23.0
Average			14.7	5.23	50.9	19.7

[a] Expressed as mg. of salicylic acid. [b] 1.00 Gm. of aspirin, or 1.12 Gm. of aluminum acetylsalicylate.

in crossover fashion 30 to 60 minutes after the noon meal with exactly 100 ml. of water. A small amount of urine was collected just prior to drug administration and served as a blank. Urine samples were then collected exactly 1 and 2 hours after drug administration. The crossover test was carried out after one week. The test subjects were instructed to eat essentially the same type and quantity of food, and at the same time, as in the previous week.

The second absorption test involved five subjects, and was initiated in the morning on empty stomachs. The test subjects were instructed to empty their bladder of overnight urine and to drink one glass of water. One hour later, the urine blank was collected and the drug was administered with 100 ml. of water. Exactly 1 hour later, the first urine sample was collected. From then on, urine samples were obtained at intervals of approximately 3 hours (except for the greater night-time interval) for about 36 hours. The time of urine collection and the volume were carefully noted.

In the case of "sustained" ASA administration, the drug was given in divided doses at 0, 3, 6, and 9 hours. The participants of the second study were cautioned not to eat until the first-hour urine sample had been collected. The interval between successive tests was 3 days or more. The drugs were administered in random order.

Analytical Method.—Salicylate in the urine was determined colorimetrically with ferric nitrate reagent (4, 5), using a Bausch and Lomb Spectronic 20 colorimeter. All readings were corrected for blank values. For first and second-hour urine samples, the zero hour blank values were used. For all other urine samples, average blank values were obtained from 24-hour urine collections of each subject.

For the determination of dissolution rates, the drug contained in aliquots of the dissolution medium was subjected to alkaline hydrolysis. The solution was then acidified, appropriately diluted with 0.1 *N* hydrochloric acid, and assayed spectrophotometrically with a Beckman model DU spectrophotometer in terms of salicylic acid.

Excretion Rates.—Individual cumulative plots of the amount of apparent salicylate excreted *vs.* time were constructed. Excretion rates were determined graphically at 1, 3, 6, 9, 12, and 15 hours over the 2-hour period having the cited time as the midpoint. The individual excretion rates were then averaged and used to construct the excretion rate *vs.* time plot.

In the case of Al.ASA, the 4-hour average excretion rate was also calculated in order to characterize the excretion rate *vs.* time curve more adequately.

Determination of Dissolution Rates.[1]—Flat-faced, one-half inch diameter tablets of the pure drugs were prepared by means of a specially modified Carver model B hydraulic press. The compression pressure was 10,000 lb. (approximately 50,000 lb. per square inch). These tablets were mounted on Plexiglas holders with the aid of paraffin wax in such a manner that only one surface of the tablet was exposed (Fig. 1). The holder with the tablet was attached to a metal shaft which, in turn, was connected to an electronically controlled precision stirring motor.[2]

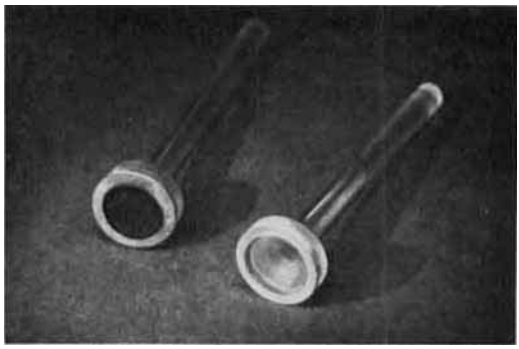


Fig. 1.—Plexiglas tablet holder for dissolution rate determinations. Empty holder (right) and holder on which a tablet has been mounted (left). The tablet shown in the picture was colored with charcoal to improve visualization.

A 200-ml. quantity of the dissolution medium was placed in a 500-ml. three-neck round-bottom flask which was immersed in a constant temperature bath adjusted to 37 ± 0.1°. After temperature equilibration was attained, the tablet attached to the Plexiglas holder was immersed in the dissolution medium to a depth of 1 inch. Under these conditions, the metal shaft did not come into contact with the solution. Just prior to immersion of the tablet, the stirrer was turned on at 555 r.p.m. The speed of rotation could be monitored by means of a continuous reading tachometer attached to a second shaft which reflected stirring speed at a fixed ratio.

Aliquots of 5 or 10 ml. were removed from the flask at appropriate intervals of time for analysis. A similar volume of dissolution medium was added each time to maintain constant volume. The tablet in its holder was weighed before and after the dissolution run as a check on the assay. The conditions of the dissolution experiment were such that the concentration of drug in the dissolution medium was a small fraction of its total solubility in order to maintain the quantity $(C_s - C)$ in the Noyes-Whitney dissolution rate expression almost constant (6, 7).

[1] The procedure is based in principle on a method developed and suggested to us by Dr. Eino Nelson, University of California, School of Pharmacy, San Francisco.

[2] Gerald K. Heller Co., Las Vegas, Nev.

TABLE II.—FIRST HOUR AND TOTAL URINARY EXCRETION OF APPARENT SALICYLATE[a] AFTER ORAL ADMINISTRATION OF ASPIRIN AND ALUMINUM ACETYLSALICYLATE

Subject	Age, yr.	Weight, lb.	One Hour After Drug Administration— ASA, 1.00 Gm.	ASA, 0.50 Gm.	Al.ASA, 1.12 Gm.[b]	Total Excretion[c] "Sustained" ASA	Al.ASA
B	26	218	26.6	20.6	13.2	652	536	458
D	21	172	18.9	10.4	7.96	545	600	484
J	33	168	27.9	8.24	13.6	538	564	532
K	22	200	19.0	9.29	1.34	399	563	358
L	21	150	39.5	17.0	4.59	547	520	374
Average			26.4	13.1	8.14	536	557	441

[a] Expressed as mg. of salicylic acid. [b] Equivalent to 1.00 Gm. of aspirin. [c] ASA = 1 Gm. given in one dose. "Sustained" ASA = 0.5 Gm. initial dose, then 0.167 Gm. every 3 hours for three doses. Al.ASA = 1.12 Gm. given in one dose.

RESULTS AND DISCUSSION

The results of the initial comparative study of Al.ASA and ASA absorption are shown in Table I. In this study, the drugs were administered after the noon meal rather than on empty stomachs so that the results may reflect absorption under conditions of normal use. The doses given were 1.00 Gm. of ASA powder and the equivalent (1.12 Gm.) of Al.ASA, respectively. The apparent salicylate excretion 1 hour after ASA administration was about three times greater than that from the aluminum salt. After 2 hours, the difference in mean amounts of salicylate excreted cumulatively was still two and one-half fold. In both instances, the differences were statistically significant ($P < 0.005$). These observations indicate that Al.ASA is absorbed much more slowly than the acid form of the drug, and that it cannot be expected to be as efficacious as ASA in the treatment of acute conditions where a reasonably rapid onset of drug action is desirable.

There is always the possibility of incomplete biological availability when a drug is absorbed from the gastrointestinal tract at an unusually slow rate since part of the drug may not be absorbed during the time in which it is in contact with the absorption sites and hence be excreted in the feces. On the other hand, poorly soluble compounds frequently afford a type of sustained action which may be desirable under certain circumstances. Accordingly, the second experiment was designed to determine salicylate excretion rate as a function of time, as well as the total amount of salicylate excreted. The rationale for using urinary excretion rates to reflect absorption rates and drug plasma levels is indicated by the following relationship (8)

$$dA/dt = K \cdot f \cdot V \cdot C \qquad \text{(Eq. 1)}$$

where A is the amount of drug excreted, K is a constant, f is the fraction of absorbed drug which is excreted eventually in the urine, V is the apparent volume of distribution, and C the drug concentration in the plasma at time t. In the case of the drugs used in this study, the values for K, f, and V are identical. Therefore, differences in excretion rate are a direct indication of differences in C, the concentration of drug in the plasma.[3] The described relationship is limited to cases where the urinary excretion rate is proportional to plasma concentration.

[3] Good comparative correlation between plasma salicylate levels and urinary excretion rates of salicylate has recently been demonstrated by Levy and co-workers (9).

In theory at least, there is the possibility that a comparative study of the urinary excretion of salicylate in the manner described may give results that are misleading. Equation 1 assumes that the ratio of the various metabolites of salicylic acid which are excreted in the urine is the same with both drugs. While this assumption appears reasonable as long as the drugs do not differ in their effect on urinary pH, it has been shown that when large doses of a drug are given, the endogenous mobilization rate of a conjugating compound may become the limiting factor in determining the rate of formation of a given metabolite (8). Thus there is a theoretical possibility that a greater fraction of the more rapidly absorbed ASA is excreted as the glucuronide of salicyclic acid than would be the case when the slowly absorbed Al.ASA is used. Since the analytical method serves to determine free salicylic acid and salicyluric acid, but not the glucuronide (10), and since the overall salicylate excretion rate is the sum of the excretion rates of each of the various metabolites and of the free drug, it is possible to obtain comparative data which do not reflect adequately the true relationship between the absorption rates or total excretion rates after administration of the two drugs.

The problem described above was overcome by the administration of ASA on a "sustained" dosage schedule. One-half gram of the drug was given at zero hour, and 0.167 Gm. was then administered every 3 hours for three doses. The dosage schedule was based on a 6-hour half-life of salicylate.[4] In this manner, any significant difference in the ratio of the excreted metabolites due to differences in absorption rate would become apparent by a comparison of the amount of salicylate (as free salicylate and salicyluric acid) excreted after single dose and "sustained" ASA administration, respectively.

The amounts excreted 1 hour after administration of ASA in single dose, of the same drug in divided doses, and of Al.ASA, as well as total amounts excreted, are listed in Table II. In this experiment, the drug was given on an empty stomach. As in the previous experiment, the first hour salicylate excretion as a result of ASA administration was about three times as high as that from Al.ASA. The difference was statistically significant ($p < 0.005$). The greater first hour absorption of both

[4] The maintenance doses, when determined by specific elimination rate constant × initial dose × hours between dosings, should actually be 0.173 Gm. A slightly lower dose (0.167 Gm.) was used as a matter of convenience in order to use an initial dose of 0.5 Gm. and provide a total of 1.00 Gm. in four doses.

drugs in the second experiment as compared with the first one is probably due to the fact that the drugs were given on empty stomachs and after the noon meal, respectively. With respect to total salicylate excretion, it is apparent that the effect of absorption rate on the ratio of the principal urinary metabolites of the drug (salicyluric acid and salicylic acid glucuronide) is negligible for the doses used in this study, since the average total apparent salicylate excretion after "single dose" and "sustained" ASA administration is essentially the same.

The total apparent salicylate excretion from Al.ASA is markedly less than that from ASA. Compared with the amount of drug excreted when ASA was given on the "sustained" dosage schedule, the biological availability of Al.ASA is only about 80% and this difference is statistically significant ($p < 0.02$). It must be concluded that Al.ASA in the form used in this study is incompletely absorbed. The degree of biological availability will of course vary depending on factors such as the particle size, the dosage form, and the experimental methods, inasmuch as the latter can affect gastrointestinal motility, pH, and gastric content. In the present study, Al.ASA was of a particle size smaller than 100 mesh, which is much more favorable than the larger particles or granules usually found in compressed tablets, as far as gastrointestinal absorption is concerned.

Figure 2 is a plot of urinary excretion rate of salicylate as a function of time. The biological half-life of salicylic acid as observed in the present study was about 6 hours. This is in excellent agreement with the 6.1-hour half-life reported by Brodie and Burns on the basis of plasma level studies (11), and is very similar to the 5 to 6-hour half-life determined by Chapman and co-workers (12), who used the urinary excretion method. The excretion rate curve obtained from "sustained" drug administration resulted in a period during which excretion rates were essentially constant. While the plateau is distinct, it is appropriate to point out that certain limitations inherent in the urinary excretion method can tend to idealize the data (13). The graphic determinations of excretion rates have been carried out with due consideration of these pitfalls and the linearity of the individual cumulative salicylate excretion curves is based, in each instance, on four experimental points. The excretion rate *vs.* time curve for Al.ASA is somewhat difficult to characterize after the 6th hour. As expected the area under the curve is smaller than that under the other two curves, reflecting the incomplete availability of the drug.

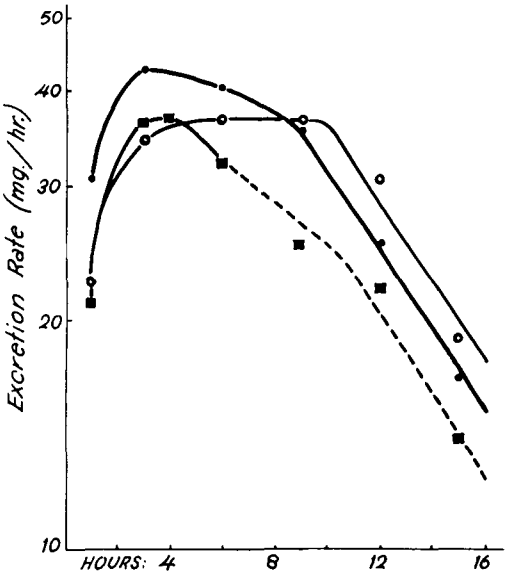


Fig. 2.—Average excretion rate of apparent salicylate as a function of time after administration of 1.00 Gm. aspirin or equivalent. ●, single dose of aspirin; ○, divided doses of aspirin; ■, single dose of aluminum acetylsalicylate.

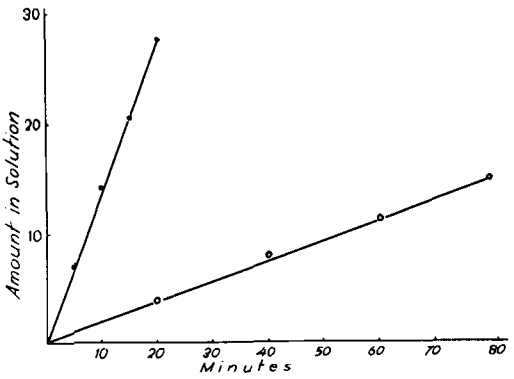


Fig. 3.—Dissolution of aspirin (●) and aluminum acetylsalicylate (○) in 0.1 *N* hydrochloric acid solution. The amount in solution is expressed as mg. salicylic acid.

Figure 3 shows the results of dissolution rate determinations using 0.1 *N* hydrochloric acid as the dissolution medium. Expressed in terms of salicylic acid, the dissolution rate of ASA is 65.1 mg./hr.-cm.^{2}, while that of Al.ASA is 8.88 mg./hr.-cm.^{2} under the conditions of the experiment. It is apparent that the dissolution rate of ASA is significantly greater than that of the aluminum salt. Similar differences were found when alkaline media were used. Since the absorption of salicylates is rate-limited by their dissolution rate in gastrointestinal fluids (5, 14), and since the dissolution rate of Al.ASA is considerably smaller than that of ASA, it is reasonable that the absorption and excretion rates of the former are also smaller than those of the latter. Dissolution of the Al.ASA used in this study in gastrointestinal fluids is evidently sufficiently slow to be incomplete, so that only part of the administered drug is eventually absorbed.

The ratio of the amounts of salicylate excreted 1 hour after administration of ASA and Al.ASA is not as unfavorable to Al.ASA as the *in vitro* dissolution data imply. This may be due to differences in the "functional" surface area of the two drugs. "Functional" surface area refers to the area actually in contact with gastrointestinal fluids and is related to particle size and shape, tendency of the particles to aggregate, and wettability of the drug solids, among others. Microscopic examination reveals that the average particle size of Al.ASA



Fig. 4.—Photomicrograph of aluminum acetylsalicylate (left) and aspirin (right) used in this study. (22 × magnification).

used in this investigation was considerably smaller than that of ASA (Fig. 4). Better quantitative correlation between dissolution and absorption rates could possibly be obtained by administering thin, highly compressed, nondisintegrating tablets of the pure drugs so that surface area would be the same in each case. The contact-irritation liability of salicylates (14,15) makes such an experiment unfeasible.

The results of this investigation indicate that Al.ASA is inherently absorbed less rapidly than ASA, and that this difference is probably due to the slow dissolution of Al.ASA as compared with ASA. Administration of Al.ASA in the form used in this study would not bring about a pharmacologic effect as rapidly as administration of ASA. The use of Al.ASA may be limited markedly by its absorption pattern and by the possibility of incomplete biological availability. While it must not be excluded that the use of certain additives could enhance sufficiently the dissolution rate of this drug to overcome the shortcomings outlined above, it is necessary to subject Al.ASA preparations to careful clinical evaluation to assure complete availability of the drug and to ascertain that it is absorbed at a rate consistent with the medicinal use of the particular product.

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Paper Chromatographic Separation and Colorimetric Estimation of the Glycosides of *Digitalis ferruginea* Seeds

By AYHAN ULUBELEN

Digitoxin, gitoxin, digoxin, digilanide A, digilanide B, digilanide C, obtained from *Digitalis ferruginea*, were successfully separated by paper chromatographic techniques and estimated colorimetrically with alkaline 3,5-dinitrobenzoic acid.

A SURVEY of the literature to date would indicate that, the seeds of *Digitalis ferruginea* have not been investigated either qualitatively or quantitatively for their cardioactive glycoside content. Considerably less attention has been given to the phytochemical investigation of the seeds of the digitalis species than of their leaves. The phytochemical investigation of the seeds of digitalis species, therefore, has been initiated as part of a long range program. In these initial studies the air-dried seeds of *Digitalis ferruginea* that were collected from Uludag-Bursa, Turkey, on October 7, 1959, were used.

A number of authors have used different reagents for the colorimetric estimation of various cardiac glycosides. The most important methods are those based on the Baljet reaction (1–6), colors given by the xanthydrol reagent (7–10),

Received April 3, 1961, from the University of Minnesota, School of Pharmacy, Minneapolis 14.
Accepted for publication May 17, 1961.
The author wishes to express her gratitude to Dr. O. Gisvold for his kind help, the laboratory hospitality of the University of Minnesota, and also to the Smith-Mundth foundation.



Fig. 4.—Photomicrograph of aluminum acetylsalicylate (left) and aspirin (right) used in this study. (22 × magnification).

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Keller-Kiliani reaction (11, 12), color reaction with 2,4-dinitrophenylsulfone (13, 14), fluorimetric methods (15, 16), methods based on the Raymond reaction (17), and color reaction with 3,5-dinitrobenzoic acid (18, 19).

An attempt was made to adapt the Baljet reaction to the small amounts of all six glycosides which were found in an extract of the seeds of *Digitalis ferruginea*. Since the intensity of the colors was very weak and not equally uniform with all six glycosides, this method was not used. The Raymond reaction, although a little more promising, was not used for the same reasons.

Finally, the method based on the color reaction given by 3,5-dinitrobenzoic acid was applied to the small amounts of glycosides and good results were obtained.

This method was described as a modification of the Raymond process by Canbäck (20) and used by Kedde (21), Rowson (18), and Lemli (19), in the estimation of digitoxin or total glycoside content of *Digitalis purpurea*.

EXPERIMENTAL

Extraction.—Before extraction, the seeds were crushed with a ball mill using flint stones. A 50-Gm. quantity of the powder, which was an oily mass, was extracted with ether. The ether was removed and the oily content of the seeds was found to be 19.4%. The ether-extracted powder, when percolated with methanol, gave a reddish-brown extract that was concentrated in a vacuum. The solid residue thus obtained was extracted with chloroform-methanol mixture, 2:1 (by volume). This extract was shaken with a 2% aqueous solution of sodium hydroxide until the aqueous layer became colorless (3 × 10 ml. sodium hydroxide was used). The chloroformic solution then was made neutral by shaking with 1% acetic acid, dried with anhydrous sodium sulfate, and evaporated in a vacuum. The residue was dissolved in a chloroform-methanol mixture, 2:1, and diluted to exactly 5 ml.

Paper Chromatography

Whatman No. 1 paper, approximately 11.5 × 36 cm., was used in the descending technique.

System I.—Method of Gisvold (22), methyl isobutyl ketone - isopropyl ether - tetrahydrofuran-formamide, 40:10:15:15 (by volume). The stationary phase formamide was applied to the paper via a 30% solution in acetone, no equilibration being necessary. The atmosphere of the tank was kept saturated by the same solvent system which was placed in a glass dish on the bottom of the tank. Development time was 4 to 5 hours.

Results.—Six very small distinct spots corresponding to digilanide A, digilanide B, digilanide C, digoxin, gitoxin, and digitoxin, respectively, were obtained with a good separation (Fig. 1). The R_f values were: digilanide C, 0.073; digilanide B, 0.126; digilanide A, 0.210; digoxin, 0.300; gitoxin, 0.490; and digitoxin 0.710.

System II.—Method of Tulus and Ulubelen (23),

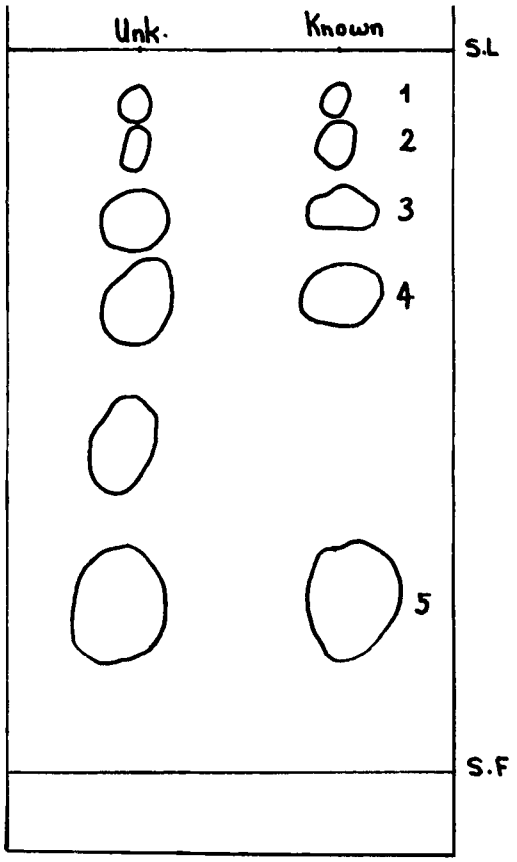


Fig. 1.—Solvent system 1. S.F, solvent front; S.L, starting line; 1, digilanide C; 2, digilanide B; 3, digilanide A; 4, digoxin; 5, digitoxin.

xylene-methyl ethyl ketone-methanol-formamide 10:10:4:0.4 (by volume). The paper was first impregnated with a 1% solution of lithium chloride and dried in the air, then the stationary phase formamide was applied to the paper by means of a 10% solution in acetone. The tank was saturated with the same solvent system; overnight equilibration was necessary. Development time was 1.5 hours.

In both systems the papers were dried 20 minutes at 110° and a sprayed Raymond's reagent was used to locate the glycosides.

Results.—The six glycosides separated very distinctly when solvent system II was used. The spots obtained by this system were somewhat larger than those obtained with solvent system I, and therefore the R_f values are different (Fig. 2). These values are as follows: digilanide C, 0.160; digilanide B, 0.210; digilanide A, 0.310; digoxin, 0.410; gitoxin, 0.610; and digitoxin, 0.780.

Preparation of the Standard Curves

A Coleman universal spectrophotometer was used in the colorimetric assays.

In order to determine the minimum time necessary for full color development and to test the stability of the color, a series of samples containing 50, 300, and 500 mcg. of digitoxin were added to 2 ml. of

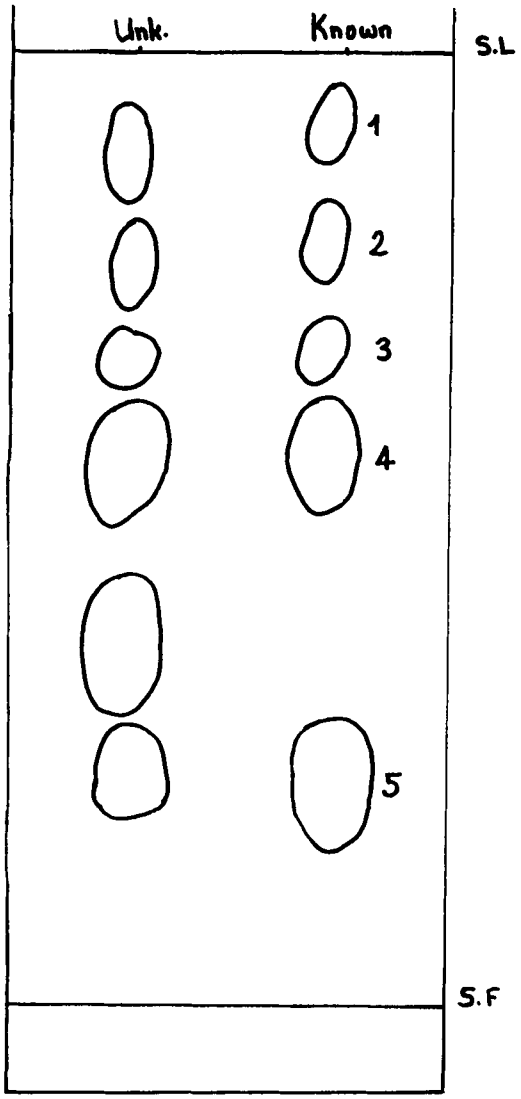


Fig. 2.—Solvent system II. S.F, solvent front; S.L, starting line; 1, digilanide C; 2, digilanide B; 3, digilanide A; 4, digoxin; 5, digitoxin.

2% 3,5-dinitrobenzoic acid (reagent I) and 1 ml. 1 *N* alcoholic solution of sodium hydroxide (reagent II), and diluted to 10 ml. with 50% alcohol. The intensities of the colors were measured at 1, 3, 5, 10, 15, 20, 30, and 40-minute intervals, and it was found that after 3 minutes the maximum color was reached. This color was stable for 30 minutes for all concentrations of digitoxin that were used. The value of color intensity for 50 mcg. was 0.08; for 300 mcg. was 0.300; and for 500 mcg. was 0.45.

The wavelength for maximum absorption was determined with 400 mcg. digitoxin and 535 mμ was found as the best wavelength (Fig. 3, Table I).

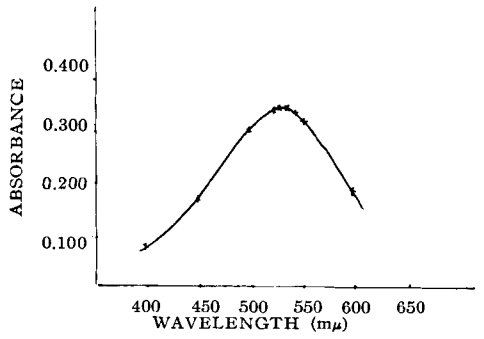


Fig. 3.—Absorption spectrum of the color produced by digitoxin with alkaline Kedde's reagent.

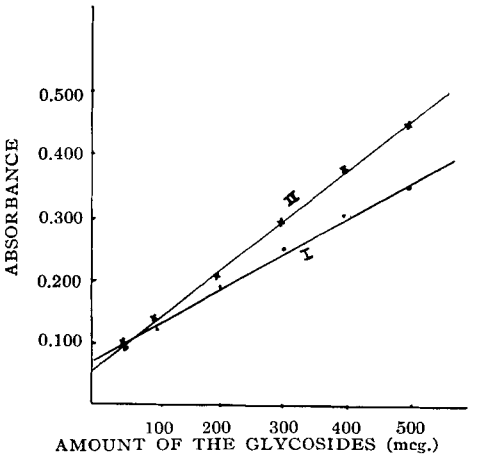


Fig. 4.—Relationship between the concentration of the glycosides and the intensity of the colors when the Kedde reaction was used. Curve I, digilanide A, digilanide B, digilanide C; curve II, digitoxin, digoxin.

TABLE II.—REPRODUCIBILITY OF THE INTENSITY OF COLOR

Sample	Absorbance 100 mcg.	Absorbance 500 mcg.
1	0.135	0.46
2	0.130	0.455
3	0.130	0.45
4	0.130	0.45

Reproducibility of the color was checked and the results are shown in Table II.

The amounts of the reagents I and II were varied, respectively, and it was found that 1.5 ml. of reagent I and 1 ml. of reagent II were sufficient for the development of maximum color when 500 mcg. of digitoxin was used in each case (Tables III and IV).

Standard curves were prepared by plotting various concentrations of digilanide A, digilanide B, digi-

TABLE I.—WAVELENGTH FOR MAXIMUM INTENSITY OF COLOR

Wavelength, mμ	400	450	500	510	520	530	535	540	550	600
Absorbance	0.08	0.17	0.30	0.31	0.33	0.335	0.34	0.32	0.31	0.18

TABLE III.—DEVELOPMENT OF COLOR WITH REAGENT I

Amount used, ml.	0.1	0.3	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Absorbance, mcg.	0.12	0.32	0.40	0.43	0.45	0.45	0.45	0.45	0.45

TABLE IV.—DEVELOPMENT OF COLOR WITH REAGENT II

Amount used, ml.	0.2	0.5	1.0	1.5	2.0	3.0
Absorbance, mcg.	0.12	0.32	0.45	0.45	0.39	0.24

TABLE V.—RELATIONSHIP BETWEEN THE CONCENTRATIONS OF VARIOUS GLYCOSIDES AND INTENSITY OF THE COLORS

	Absorbance				
Glycosides, mcg.	Digilanide C	Digilanide B	Digilanide A	Digoxin	Digitoxin
50	0.09	0.09	0.10	0.09	0.095
100	0.125	0.12	0.12	0.13	0.13
200	0.190	0.185	0.190	0.205	0.205
300	0.250	0.240	0.240	0.290	0.300
400	0.300	0.300	0.300	0.370	0.360
500	0.340	0.340	0.340	0.445	0.450

TABLE VI.—PERCENTAGES OF VARIOUS GLYCOSIDES IN THE SEEDS OF *Digitalis ferruginea*

Glycosides	Absorbance[a]	mcg. in 60 μl.	mg. in 5 ml.	% in Drug[b]
Digilanide C	0.147	140	11.666	0.02333
Digilanide B	0.168	175	14.58	0.02916
Digilanide A	0.168	175	14.58	0.02916
Digoxin	0.147	115	9.583	0.01916
Gitoxin	0.177	155	12.91	0.02582
Digitoxin	0.194	180	16.00	0.03200

[a] Average of 4 determinations. [b] These amounts were calculated from the relationship between the concentration and absorbance which is shown in Fig. 4. Total amount of glycosides is 0.1579%.

lanide C, digitoxin, and digoxin *versus* the intensities of the colors over a range 50–500 mcg. The concentrations were found proportional to the absorptions within this range. Table V gives the results and Fig. 4 shows the curves.

Table V and Fig. 4 show that digilanide A, B, and C give the same curve (curve I) and digitoxin and digoxin give curve II. There is no reason why gitoxin should not also give the same curve.

Analysis of Glycosides of the Seeds

Known amounts of glycosides (150 mcg. each) were applied to the paper and after development according to the methods I and II, respectively, the areas on the paper containing glycosides were cut out and extracted by means of 10 × 2 ml. methanol. The solvent was evaporated and the color was developed as described previously. The amounts of the known glycosides were measured against a blank, which was prepared with the reagents. The amount that was applied to the paper was always recovered. Then exactly 60 μl. of the chloroformic solution of the crude drug, which was previously prepared, was applied to the paper. After development, the areas on the paper containing the glycosides were cut out and extracted by means of 10 × 2 ml. methanol and the colors were developed and measured as described above. Results are shown in Table VI.

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Interactions of Xanthine Molecules with Bovine Serum Albumin

By M. L. EICHMAN†, D. E. GUTTMAN, Q. VAN WINKLE, and E. P. GUTH

The binding of a number of structurally related xanthine compounds by bovine serum albumin was investigated in an attempt to elucidate some of the structural specificities of the interaction. The study included caffeine, theophylline, 8-nitrotheophylline, 8-chlorotheophylline, theophylline-7-acetic acid, 8-methoxycaffeine, 8-ethoxycaffeine, 8-chlorocaffeine, 8-methylcaffeine, theobromine, 8-nitrotheobromine, uric acid, 1,3,7-trimethyluric acid, uracil, and 1,3-dimethyluracil. Under the conditions employed, interactions between the protein and uric acid, uracil, and dimethyluracil could not be detected. The remaining compounds were bound to varying degrees and evidence was obtained to indicate that a single site on the protein was responsible for the major portion of the binding. Anionic species were generally found to possess much stronger interactive tendencies than those which had no formal charge.

THE ABILITY of serum proteins, particularly albumin, to reversibly bind numerous small molecules has been recognized for many years (1). The majority of earlier investigations were of a qualitative nature. Only in recent years have attempts been made to quantitate the results and to explain the nature of the equilibria and forces involved in the interactions (2, 3). Concomitant with advances in the theoretical aspects of these interactions, there has been an increase in the study of the associations of drug molecules with proteins. Steroid hormones (4), barbiturates (5), sulfonamides (6, 7), penicillins (8), salicylates (9), and other therapeutic agents have been shown to interact with serum albumins.

Goldstein (1), and Brodie and Hogben (10) have discussed the significance of such behavior in the transport, deposition, and elimination of drugs in the body. In addition, it appears that the pharmacologic activity of many medicinal agents is intimately related to their abilities to combine with specialized functional proteins. Elucidation of the factors involved in such interactions may thus contribute to the understanding of drug action.

The interaction of xanthine compounds with components in blood serum had been noted in the early investigations of Pak (11) and Aiello (12). More recently, Schack and Waxler (13) demonstrated that theophylline can be bound by the plasma proteins of rabbits. In the present study, the bindings of caffeine and a number of structurally related compounds by bovine serum albumin (BSA) were quantitatively investigated in an attempt to determine the dependency of the interaction on structural and environmental variables. The compounds employed are listed in Table I. The results will show that the interaction of xanthine derivatives with BSA was dependent on the nature of substituents at the 1, 7, and 8 positions, the acid strength of the compound, and on the hydrogen-ion concentration of the system.

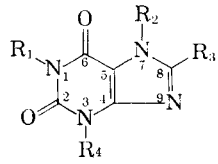
EXPERIMENTAL PROCEDURE

Materials.—Bovine serum albumin, fraction V (BSA), purchased from Armour Laboratories (lots T16812 and U17912), was used throughout this study. Electrophoretic analysis of lot T16812 indicated an albumin content of 97.5%. A molecular weight of 69,000 was assumed. Both lots of albumin were found to exhibit similar binding tendencies for caffeine. Reagent grade mono, di, and trisodium phosphate salts were employed to buffer the solutions and to obtain an equilibrium ionic strength of 0.16. The xanthine derivatives were either purchased or prepared by standard synthetic procedures. All xanthine and albumin solutions were prepared imediately prior to use.

Dialysis Studies.—Equilibrium dialysis experiments, essentially as described by Hughes and Klotz (14), were used to investigate the binding of xanthines by BSA. Ten-milliliter samples of albumin in phosphate buffer were accurately pipetted into bags prepared from Visking cellophane casing. The bags were placed into tubes, each containing a different concentration of the xanthine derivative. The range of concentrations employed was from 5×10^{-5} M to 2×10^{-2} M. A series of control tubes in which buffer solution was used in place of protein solution was similarly prepared. The bags prevented the passage of albumin into the external solutions, but were permeable with respect to the xanthine molecules. Binding of xanthines by the dialysis membrane was found to be insignificant. Equilibrium was established by gentle mechanical

Received September 6, 1960, from The Ohio State University, College of Pharmacy, Columbus 10.
Accepted for publication February 20, 1961.
Abstracted from a dissertation presented to the Graduate School of The Ohio State University by Martin L. Eichman, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
Presented to the Scientific Section, A. PH. A., Washington, D. C., meeting, August 1960.
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TABLE I.—STRUCTURES AND MOLAR ABSORPTIVITIES OF SOME XANTHINE DERIVATIVES



Compound	R_1	R_2	R_3	R_4	λ max., mμ	Molar Absorptivity × 10^{-4}
Caffeine	CH_3	CH_3	H	CH_3	272	0.956
Theophylline	CH_3	H	H	CH_3	272	1.020
Theobromine	H	CH_3	H	CH_3	273	1.008
8-Methoxycaffeine	CH_3	CH_3	OCH_3	CH_3	281	1.326
8-Ethoxycaffeine	CH_3	CH_3	OCH_2CH_3	CH_3	281	1.365
8-Methylcaffeine	CH_3	CH_3	CH_3	CH_3	275	1.148
8-Chlorocaffeine	CH_3	CH_3	Cl	CH_3	277	1.154
8-Nitrotheophylline	CH_3	H	NO_2	CH_3	387	1.196
8-Chlorotheophylline	CH_3	H	Cl	CH_3	277	1.310
Theophylline-7-acetic acid	CH_3	CH_2COOH	H	CH_3	274	0.975
8-Nitrotheobromine	H	CH_3	NO_2	CH_3	273	1.218
Uric acid	H	H	OH	H	291	1.136
Trimethyluric acid	CH_3	CH_3	OH	CH_3	295	1.636

shaking at 9 ± 1° for 30 hours. All xanthine compounds were studied at pH 6.85. In addition, the interactions of caffeine, theophylline, 8-nitrotheophylline, and 8-chlorotheophylline were also investigated over a wide pH range. At equilibrium, the external solutions were analyzed for the xanthine derivative and the exact pH determined from measurements on the albumin solution in each tube. Solutions of 0.4% albumin were employed in studies of the binding of 8-chlorotheophylline and 8-nitrotheophylline. Albumin solutions, 2%, were used in all other studies.

Analytical Methods.—The concentration of xanthine derivative in each solution was determined spectrophotometrically with a Beckman model DU spectrophotometer. For each compound, the wavelength of maximum absorption was employed. The experimentally determined molar absorptivities are listed in Table I. Appropriate dilutions were made where necessary so that all spectrophotometric readings were recorded below 0.8 absorbance units. Suitable blanks were employed to correct for spectral contributions of dialyzable components in the albumin and from soluble substances in the dialysis membrane. The albumin concentrations were determined spectrophotometrically at 278 mμ. The molar absorptivity of albumin was determined to be 4.603×10^4. This value was not corrected for nonalbumin impurities present in the protein. The pH values of the protein solutions were determined at 25° with a Beckman model G pH meter after each dialysis experiment.

THEORY AND RESULTS

A cursory examination of the data reveals marked differences in the tendencies of the compounds investigated to associate with BSA. This is illustrated in Table II, where the percentage of each xanthine bound by a 2% albumin solution is tabulated. The values are seen to range from 3% for the total amount of theobromine bound, to 98% for 8-nitrotheophylline and 8-chlorotheophylline. These figures were determined from dialyses of 10 ml. of 1×10^{-4} *M* solutions of each xanthine against 10 ml. of BSA solution.

TABLE II.—PERCENTAGE OF EACH XANTHINE DERIVATIVE BOUND BY 2% ALBUMIN SOLUTIONS

Compound	% Bound
Caffeine	17
Theophylline	20
Theobromine	3
8-Chlorocaffeine	58
8-Methoxycaffeine	67
8-Ethoxycaffeine	46
8-Methylcaffeine	40
8-Chlorotheophylline	98
8-Nitrotheophylline	98
Theophylline-7-acetic acid	44
8-Nitrotheobromine	3
Uric acid	6
1,3,7-Trimethyluric acid	83
Uracil	Negligible
1,3-Dimethyluracil	Negligible

In view of these results, it was considered desirable to characterize quantitatively each of the systems studied by one or more equilibrium constants and to determine the number of sites on the protein molecule participating in the interactions. In this way, a clearer understanding of the interactions may be achieved so that logical comparisons between compounds can be made. The mathematical analysis of binding data of the type presented here has been described and discussed by Klotz (2, 15) and Scatchard (16). If only one class of sites on the protein is responsible for the binding, the number of moles of small molecule bound per mole of protein can be related to the concentration of the free small molecule by

$$r = \frac{nk(\mathrm{A})}{1 + k(\mathrm{A})} \qquad \text{(Eq. 1)}$$

where r = moles of small molecule bound per mole of protein, n = number of binding sites available on each protein molecule, k = association constant for

the reaction, and (A) = concentration of unbound small molecule. The values of n and k can be graphically determined by plotting the data in accordance with a rearranged form of Eq. 1

$$r/(A) = nk - rk \qquad \text{(Eq. 2)}$$

A plot of $r/(A)$ *vs.* r will result in a straight line of slope $-k$ and ordinate and abscissa intercepts of nk and n, respectively.

When both the protein and the small molecule possess a formal charge, an electrostatic correction factor must be applied. In such cases, a modified formula, as suggested by Scatchard (16), can describe the system

$$[r/(A)]\ f = nk - rk \qquad \text{(Eq. 3)}$$

where $f = e^{2wZ_PZ_A}$, Z_P = the charge on the protein, Z_A = the charge on the small molecule, and w = an electrostatic parameter. Tanford, *et al.* (17), evaluated w to be equal to 0.026 at $\Gamma/2 = 0.16$. At pH 6.85, Z_P was taken as -7 and Z_A was -1 for the anionic xanthines and 0 for the neutral molecules. If the xanthine molecule is uncharged, f is unity, and Eq. 3 becomes identical to Eq. 2.

A general description of the system where two or more classes of binding sites on the macromolecule participate in the interaction is given by Eq. 4. In this case, each class of sites will possess a different affinity for the small molecule

$$r/(A) = \frac{n_1k_1}{1 + k_1(A)} + \frac{n_2k_2}{1 + k_2(A)} + \ldots + \frac{n_ik_i}{1 + k_i(A)} \qquad \text{(Eq. 4)}$$

Such behavior is indicated by curvature of the $r/(A)$ *vs.* r plot. It can be shown that the abscissa and ordinate intercepts in this case will be Σn and Σnk, respectively. In order to evaluate constants from nonlinear data of this type, a method described by Scatchard, *et al.* (18), and Karush (19) was employed, in which only two classes of sites on the protein were assumed to account for the binding. Our results indicated Σn to be equal to three. The data were therefore evaluated for several compounds with the assumption that the two classes of binding sites contain one and two groupings, respectively. The curves calculated on this basis agree well with those drawn through the experimental points. The values for k_1 and k_2 are listed in Table III. Where no values are given for k_2, secondary interactions have been found to be negligible and the bindings are therefore adequately represented by k_1. This was the case for caffeine and its 8-substituted derivatives. Their k values were obtained by linear extrapolation to the ordinate axis in the $r/(A)$ *vs.* r plots, as illustrated in Fig. 1. In contrast, secondary interactions were quite prominent in the case of 8-chlorotheophylline, theophylline-7-acetic acid, and trimethyluric acid. The values for both k_1 and k_2 were estimated for these compounds.

Table III.—Association Constants for the Binding of Xanthine Derivatives by Albumin

Compound	$n_1 = 1$, $k_1 \times 10^5$	$n_2 = 2$, $k_2 \times 10^{-4}$	pH
Caffeine	0.0154	0.003	4.80
Caffeine	0.0168	...	6.85
8-Methylcaffeine	0.0520	...	6.85
8-Ethoxycaffeine	0.0750	...	6.85
8-Chlorocaffeine	0.125	...	6.85
8-Methoxycaffeine	0.158	...	6.85
Theophylline	0.0160	0.004	5.80
Theophylline	0.0161	0.009	6.85
Theophylline	0.0198	0.021	7.23
Theophylline	0.0253	0.037	7.85
8-Nitrotheophylline	2.64	1.27	6.85
8-Chlorotheophylline	3.07	1.10	6.85
Theophylline-7-acetic acid	0.0592	0.061	6.85
Theobromine	0.0024	...	6.85
8-Nitrotheobromine	0.0020	...	6.85
Uric acid	0.0075	...	6.85
1,3,7-Trimethyluric acid	0.344	1.21	6.85
Uracil	Negligible	...	6.85
1,3-Dimethyluracil	Negligible	...	6.85

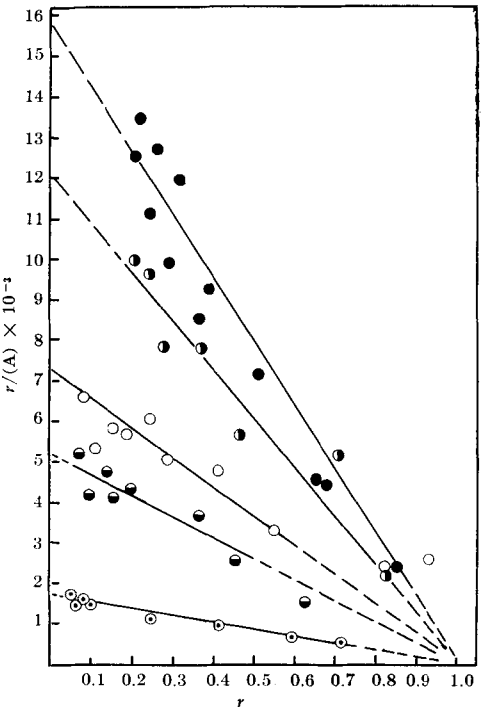


Fig. 1.—The binding of caffeine and 8-substituted caffeines by BSA. ●, 8-methoxycaffeine; ◑, 8-chlorocaffeine; ○, 8-ethoxycaffeine; ◒, 8-methylcaffeine; ⊙, caffeine.

DISCUSSION

The ability of caffeine to form complexes with a large number of structurally different small molecules has been well documented (20, 21). On this basis, it was expected that several different groupings on the albumin macromolecule might serve as binding sites for caffeine. From the study illustrated in Fig. 2, it was apparent, however, that the principal interaction with BSA involved only one site on the protein. The low magnitude of k_2 indicated that secondary associations were of minor significance. Information pertaining to the nature of the primary site was adduced from the influence of pH on the interaction. This is illustrated in Fig. 3. The

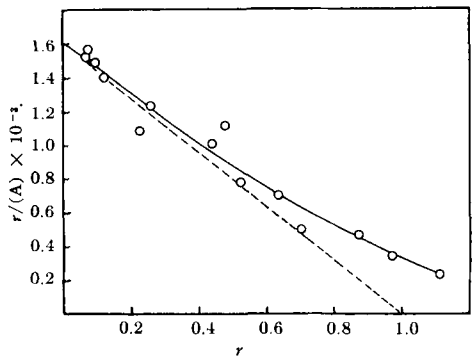


Fig. 2.—The interaction of caffeine with BSA at pH 4.80. – – –, primary interaction; ——, primary plus secondary interactions.

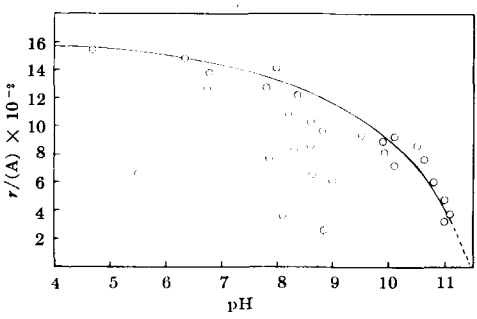


Fig. 3.—The effect of pH on the binding of caffeine.

gradual decrease in binding which occurred between the pH values of 4.8 and 8.5 may be the result of slight changes in the structure of the albumin. The marked decrease in association above pH 8.5 may be rationalized either by assuming that a specific interaction occurred between caffeine and a protonated site on the protein or by considering a binding site to be involved which was either blocked or abolished by a configurational change which resulted from the neutralization of a protonated group on the protein. Ionizable substituents on albumin include carboxyl, imidazole, amino, phenolic, guanidino, and sulfhydryl groups. Since the protonated • amino groups of lysine dissociate in the pH range where the major decrease in binding occurred, it is quite possible that neutralization of such a group is related to the marked decrease in the interaction.

The xanthine precursors, uracil and 1,3-dimethyluracil, as well as the dimethylxanthines, theophylline and theobromine, were investigated for binding activity in order to elucidate further the factors involved in the interaction. It was initially considered that the pyrimidinedione nucleus might be responsible for the binding. However, investigations with uracil and dimethyluracil revealed that neither compound was capable of interaction. The interactions of caffeine and theophylline as compared with that of theobromine emphasize the importance of N_1 substitution. The strengths of binding exhibited by the former compounds were considerably greater than that of the latter. It appears therefore, that not only are both rings required in order for significant binding to occur, but, in addition, substitution at the N_1 position is also a necessary prerequisite.

In contrast to caffeine, theophylline exhibited an increase in interaction as the pH was raised above 4.8, as shown in Fig. 4. From the association constants, k_1 and k_2, listed in Table III for several pH values, it is seen that this increase is the result of both greater primary and secondary interactions. The differences in the binding behaviors of caffeine and theophylline can be attributed to the ionization of theophylline (pKa 8.7). The fraction of theophylline in its anionic form becomes significant within the pH region where the rise in association occurred. It appears, therefore, that the negatively charged form of theophylline is more strongly bound than the uncharged molecule. At higher pH values the sharp decrease in the binding of theophylline parallels that observed with caffeine and is most probably related to the release of a proton from a specific acidic group on the protein.

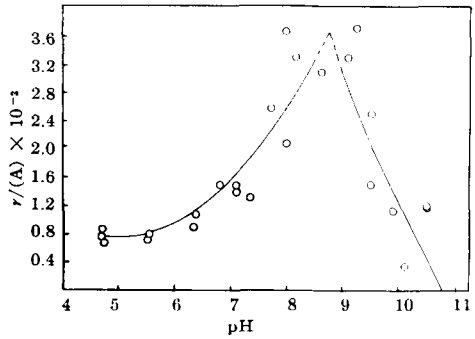


Fig. 4.—The effect of pH on the binding of theophylline.

The interactions of 8-substituted caffeines are graphically represented in Fig. 1. It is seen that in all cases association appears to involve one site on the protein. Marked differences in binding strength are, however, apparent. For example, the association constant for the 8-methoxycaffeine–BSA interaction is approximately ten times that found for the analogous caffeine system. Although it is difficult to interpret these results, the involvement of both steric and electronic factors are considered likely. Greater van der Waals attraction forces, resulting from the larger molecular size of the small molecule may account for the increased binding of the substituted derivatives. Another possible explanation lies in the ability of the small molecule to fit into a receptor site on the protein. Lumry and Eyring (22) have indicated that the molecule which would provide the best fit for the binding site would be expected to produce the strongest interaction. The 8-substituted caffeines, because of their larger sizes, may be more capable of filling the available space.

The interactive tendencies of 8-chloro and 8-nitrotheophylline at pH 6.85 were considerably greater than those of other compounds investigated. The binding curve for 8-chlorotheophylline is shown in Fig. 5 where a theophylline curve is also plotted for comparative purposes. A similar curve was obtained for the 8-nitrotheophylline–BSA system. The marked curvature is indicative of rather

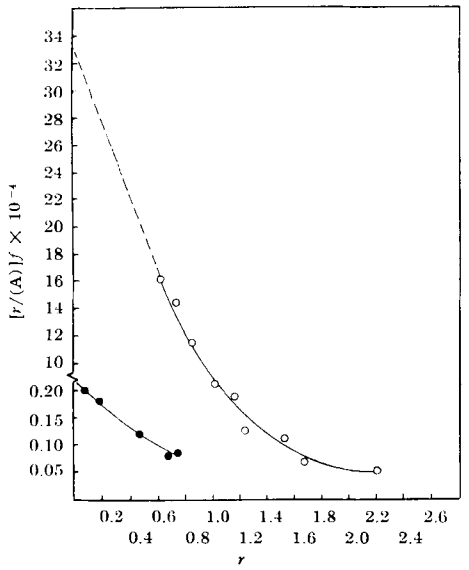


Fig. 5.—The bindings of 8-chlorotheophylline and theophylline by BSA at pH 6.85. O, 8-chlorotheophylline; ●, theophylline.

strong secondary interactions. It may be noted from Table III that the association constants for 8-chlorotheophylline and 8-nitrotheophylline at pH 6.85 are larger than those of other compounds investigated by magnitudes ranging from approximately ten to several hundred.

It is significant in this respect to compare the degrees of ionization at pH 6.85 of the various compounds investigated. It is found that 8-chlorotheophylline (pKa = 5.5) and 8-nitrotheophylline (pKa = 3.6) were completely ionized at this pH value. Furthermore, studies which are illustrated in Fig. 6 demonstrate a marked relationship between the degree of association with BSA and the ionization characteristics of the two compounds. 8-Nitrotheophylline, for example, was completely ionized throughout the pH range investigated and its interactive tendencies remained relatively constant between pH values of 4.8 and 8.5. In contrast, the fraction of 8-chlorotheophylline which existed in the

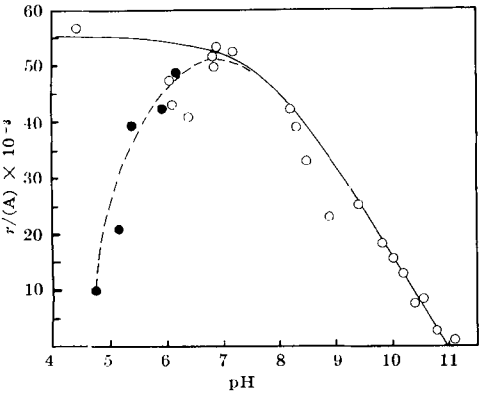


Fig. 6.—The effect of pH on the binding of 8-nitrotheophylline and 8-chlorotheophylline. O, 8-nitrotheophylline; ●, 8-chlorotheophylline.

ionized form increased in the pH range of 4.8 to 6.5 and was the predominant specie at pH values above this. Its association with BSA exhibited a similar pH dependency. Both compounds exhibited the diminution in binding at higher pH values which seems to be a characteristic of xanthine derivative-BSA interactions.

It can be concluded from the studies with theophylline and its 8-substituted derivatives that the anionic forms possess much greater binding tendencies than their nonionic counterparts. The basic nature of the association does, however, appear to be the same. The primary interactions of both types appear to involve one grouping on the protein and in both cases marked inhibition of binding occurred at pH values above approximately 8.5.

In view of these results, it was of interest to investigate the interactions of other anionic xanthine derivatives. Studies were therefore conducted with theophylline-7-acetic acid, trimethyluric acid, and uric acid. The binding of theophylline-7-acetic acid was observed to be greater than that of theophylline but significantly less than that of the 8-substituted theophyllines. This indicated that factors other than the mere presence of a negative charge determine the affinity with which the xanthine molecule is bound by the protein. This is substantiated by the results of studies with uric acid and trimethyluric acid. These compounds, too, are ionized at pH 6.85; their negative charge occurring at N_9. The interaction of uric acid was slight. On the other hand, trimethyluric acid was bound to a greater degree than the 8-substituted caffeine derivatives. Here too, however, its association constant was considerably smaller than those for the 8-substituted theophyllines.

On the basis of these results it can be stated that, although negatively charged species appeared to be more strongly bound to albumin than neutral molecules, the position of charge is of great significance. Theophylline and its 8-substituted derivatives ionize through removal of the proton from N_7. In view of the greater electronegativity of oxygen as compared to nitrogen, Cavalieri, *et al.* (23), have suggested that the negative charge probably resides on the oxygen on C_6. This shift in position of charge is facilitated by resonance in the molecule. It is consistent with the results to hypothesize that this oxygen participates directly in the binding reaction; perhaps through hydrogen bond formation with a protonated grouping on the protein. The strength of such a bond would be substantially increased if the oxygen were to possess a negative charge. In addition, it may be noted that a methyl substituent on the N_1 position will increase the electron density around the oxygen through an inductive effect.

Although the information which has been provided by this preliminary study is insufficient to characterize completely the interaction between BSA and xanthine derivatives, some interesting observations have been made. Of particular interest is the suggested involvement of only one grouping on the protein molecule. There is evidence to suggest that this grouping is an ϵ amino group of lysine. Since it has been reported that albumin contains 22 lysine residues, it can be speculated that the configuration of the macromolecule is such that only one of the lysine side chains is in an environment which will permit significant interaction with this family of

small molecules. Further studies are being undertaken to substantiate this hypothesis and to determine the nature of the forces which are responsible for the association.

The influence which slight structural modification of the small molecule has on the degree and strength of binding is also rather intriguing. The large differences which were found among the 8-substituted caffeines are particularly interesting. Variations in biological half-lives and in pharmacological activities might be partially explainable on this basis and studies are planned to test this possibility.

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Effects of Tranquilizers on Bacterial Toxemias III

Chlorpromazine

By LEO GREENBERG and JAMES W. INGALLS

Previous papers from this laboratory have indicated the role of reserpine and meprobamate in overwhelming bacterial toxemias experimentally induced in laboratory animals. The present study was primarily concerned with chlorpromazine. In rats and mice, pretreatment with chlorpromazine was shown to prolong significantly the survival of animals inoculated with lethal doses of tetanus or botulinus toxins. Similar prolongations following chlorpromazine pretreatment were demonstrated with *E. coli, S. typhosa,* and *S. marcescens* endotoxins. At identical doses, neither hydroxyzine nor methaminodiazepoxide pretreatment consistently provoked similar responses to toxin challenges.

SINCE 1957, our laboratories have been primarily concerned with the role of tranquilizing drugs in experimental stress induced in laboratory animals through the use of cultures of virulent microorganisms and by various potent microbial products, both of exotoxic and endotoxic type. We have found that reserpine exerted a highly significant life-prolonging effect in tetanus and botulinus exotoxemias and pneumococcal septicemia of mice (1). Similar prolongation of survival time in exotoxemias was found with meprobamate treatment in rats of both sexes as well as mice, although no influence on pneumococcal septicemia was observed (2). Further, the life-prolonging properties of meprobamate were shown to extend to animals inoculated with lethal doses of purified lipopolysaccharide endotoxins of *Escherichia coli* and *Salmonella typhosa* (2). In all cases, the prolongation of survival time was associated with a specific set of experimental conditions, namely the administration of a relatively large dose of tranquilizer approximately 1 hour prior to challenge with an overwhelmingly lethal dose of the toxic agent.

The present study summarizes the results of our investigation of chlorpromazine within this framework of bacterial stress. In the case of chlorpromazine, unlike that previously found with reserpine and meprobamate, the literature is replete with references, both clinical and experimental, indicating an influence of the drug in disease processes. Unfortunately, the direction and magnitude of the influence reported has sometimes been contradictory. Thus, it has been noted that chlorpromazine shortened survival time from pneumococcal (3, 4) or *Salmonella*

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enteritidis infection (2, 5), but did not influence *Salmonella typhimurium* infection (2). It shortened survival time from nasally inoculated streptococci but not from intraperitoneally inoculated streptococci (3). It either depressed (6,7) phagocytosis or enhanced (8) phagocytosis, depending upon the technique used. It had no apparent influence on *Plasmodium berghei, Trypanosoma Congolese,* or *Trypanosoma brucei* infections (3). In low doses, the drug shortened survival from *Trypanosoma cruzi* infections, but did not do so in moderate doses (9). In high doses, chlorpromazine treatment increased survival from *Trypanosoma evansi* infection (9).

In experimentally induced endotoxemias, chlorpromazine treatment is usually reported to exert a favorable influence on survival time (10–14) although no protection against *Aerobacter aerogenes* endotoxin was found (13). With *Salmonella typhosa* endotoxin, Noyes, *et al.* (13), have reported prolongation of survival time with chlorpromazine treatment in two experiments, and decreased survival time in one experiment. Little information exists concerning the role of the drug in exotoxemias. An increased mortality from *Clostridium perfringens* toxin but no influence on botulinus, tetanus, or *Pasteurella pestis* intoxication with chlorpromazine treatment has been noted (13).

Our investigation has been concerned primarily with the influence of chlorpromazine on exotoxemias and in stress induced by purified endotoxins. In almost all cases, hydroxyzine[1] and methamiondiazepoxide[2] were used as comparisons to chlorpromazine since, to the best of our knowledge, no previous study has investigated the possible role of these two potent tranquilizers in bacterial stress.

EXPERIMENTAL

Materials and Methods.—The animals used in this study were 200–300-Gm. male and female CFN rats and 20–25-Gm. CF_1 male mice (Carworth Farms), caged in small groups and kept in the thermostatically controlled animal house for several days prior to use. In all experiments, food was withheld after the toxic challenge but water was allowed *ad libitum.*

All drugs[3] were diluted with sterile, isotonic saline. In all experiments, matched groups of 10 animals were used. Tranquilizer injections were made intraperitoneally in a volume of 0.2 ml. 1 hour prior to the toxin challenge. Control animals received 0.2 ml. of sterile saline injection. The control and experimental animals in any given series were inoculated within a few minutes of one another.

Clostridium botulinum type A toxin[4] diluted with two parts of glycerol and having a mouse LD_{50} of approximately 5×10^6/ml. was used as a challenge, as was tetanus toxin[5] in 0.85% saline with 0.3 *M* glycine, an Lf. titer of 870/ml., and a mouse LD_{50} of 112 million. Toxins were stored in the refrigerator, the botulinus material at −20° to maintain potency.

Escherichia coli, Salmonella typhosa, and *Serratia marcescens* endotoxins were purchased as powdered lipopolysaccharides from Difco Laboratories. Powdered *Salmonella equi* endotoxin was graciously sent by Dr. Otto Westphal of the Dr. A. Wander Forschungsinstitut of Freiburg-Zahringen, Germany. All toxins were diluted with sterile saline and warmed to body temperature prior to intraperitoneal injection in a volume of 0.1 ml.

For the single diphtheria experiment, large male guinea pigs were purchased locally. Diphtheria toxin[5] partially purified from broth, with an LD_{50} of approximately 3×10^5/ml. was used as the challenge.

RESULTS AND DISCUSSION

The data comparing chlorpromazine with hydroxyzine and methaminodiazepoxide in various exotoxic and endotoxic conditions are summarized in Table I. From these data, a consistent pattern of response with chlorpromazine pretreatment is evident. In all cases, a moderate dose of the drug administered intraperitoneally 1 hour prior to challenge either by exotoxins or endotoxins resulted in a marked increase in survival time. This response is apparently unrelated to the general tranquilizing properties of chlorpromazine since neither of the other two tranquilizing drugs tested, hydroxyzine and methaminodiazepoxide, showed similar propensities.

Although the data are not included in Table I, preliminary studies showed that marked increases in survival time were not induced by relatively small doses of chlorpromazine (on the order of 2.5 mg./Kg.); and, as in our previous findings with reserpine (1) and meprobamate (2), no influence on survival was noted if the drug was administered after the toxin challenge.

It will be noted that of the three major exotoxins of bacterial origin, only tetanus and botulinus toxins were employed in this study. Mice and rats are notoriously refractory to diphtheria toxin, and the effects of various tranquilizers, including chlorpromazine, on survival from diphtheric intoxication in guinea pigs has been presented earlier (15). It may be of interest to mention that since this earlier study did not include data on methaminodiazepoxide, 11 guinea pigs were pretreated with 25 mg./Kg. of the drug prior to challenge with diphtheria toxin. Eleven untreated control animals showed a mean death time of 12.9 ± 0.5 hours while the treated animals died in 13.1 ± 0.5 hours, a difference of no statistical significance.

In speculating on the mode of action by which chlorpromazine manifests its antitoxic properties,

[1] Marketed as Atarax by J. B. Roerig and Co.
[2] Marketed as Librium by Roche Laboratories.
[3] Chlorpromazine supplied as Thorazine (Smith Kline and French), hydroxyzine supplied as Atarax (J. B. Roerig and Co.), methaminodiazepoxide supplied as Librium (Roche Laboratories), and reserpine supplied as Serpasil (Ciba) through the courtesy of their manufacturers.
[4] Supplied through the courtesy of Matteo Cardella, Immunology Branch, U. S. Army Biological Warfare Laboratories, Fort Detrick, Frederick, Md.
[5] Supplied through the courtesy of Dr. H. A. Dettwiler, Eli Lilly and Co., Indianapolis, Ind.

TABLE I.—EFFECT OF CHLORPROMAZINE (25 MG./KG.), HYDROXYZINE (25 MG./KG.), AND METHAMINODIAZEPOXIDE (25 MG./KG.) ON SURVIVAL TIME FROM BACTERIAL TOXINS IN MATCHED GROUPS OF RATS AND MICE

Animal	Treatment	Survival, min. ± S.E.	Significance
	Botulinus Exotoxin (1–10)		
Mice (Male)	Control	82 ± 3	....
	Hydroxyzine	78 ± 3	None
	Methaminodiazepoxide	84 ± 3	None
	Chlorpromazine	102 ± 6	$P < 0.01$
Rats (Female)	Control	240 ± 11	....
	Hydroxyzine	254 ± 17	None
	Methaminodiazepoxide	309 ± 6	$P < 0.001$
	Chlorpromazine	459 ± 14	$P < 0.001$
	Tetanus Exotoxin (1–10)		
Mice (Male)	Control	94 ± 2	....
	Hydroxyzine	112 ± 21	None
	Methaminodiazepoxide	123 ± 20	$P > 0.2$
	Chlorpromazine	150 ± 15	$P < 0.01$
Rats (Male)	Control	629 ± 11	....
	Hydroxyzine	687 ± 26	None
	Methaminodiazepoxide	647 ± 13	None
	Chlorpromazine	994 ± 71	$P < 0.001$
	Serratia marcescens Endotoxin (1–50)		
Mice (Male)	Control	995 ± 52	....
	Hydroxyzine	997 ± 77	None
	Methaminodiazepoxide	1041 ± 54	$P > 0.5$
	Chlorpromazine	1387 ± 57	$P < 0.001$
	Escherichia coli 0127:B8 Endotoxin (1–50)		
Mice (Male)	Control	806 ± 40	....
	Hydroxyzine	901 ± 59	None
	Methaminodiazepoxide	850 ± 52	None
	Chlorpromazine	1136 ± 38	$P < 0.001$
	Salmonella equi Endotoxin (1–50)		
Mice (Male)	Control	963 ± 58	....
	Chlorpromazine	1024 ± 65	$P > 0.4$
	Escherichia coli 026:B11 Endotoxin (1–50)		
Mice (Male)	Control	622 ± 38	....
	Chlorpromazine	1152 ± 55	$P < 0.001$
	Salmonella typhosa Endotoxin (1–50)		
Mice (Male)	Control	624 ± 35	....
	Chlorpromazine	1122 ± 78	$P < 0.001$

we noted with interest a suggestion by Boroff (16) that both chlorpromazine and reserpine release serotonin into the peripheral circulation and that both of these drugs should antagonize toxins since serotonin itself has been shown to exert a powerful antitoxic effect in laboratory animals (16, 17). If the underlying assumption concerning serotonin release is true, this is an attractive hypothesis, and our results with chlorpromazine would not be surprising. However, Berger, *et al.* (18), were not able to demonstrate any increase in serotonin metabolites after chlorpromazine administration, although the response was marked after reserpine administration.

Moreover, it has been reported recently (19) that a dose of reserpine known to release considerable serotonin into mouse circulation was ineffective in altering the lethality of an *Escherichia coli* endotoxin. We therefore undertook a large-scale experiment to clarify this point. Using 20 matched groups of animals, high and low doses of reserpine administered at different times prior to challenge with four different *E. coli* lipopolysaccharide preparations were evaluated. Death times were recorded hourly around the clock until the conclusion of the experiment. In no case did reserpine treatment have any significant effect, either for better or worse, on the lethality of the various endotoxins.

Until further information is available, we can only conclude that the relationships among chlorpromazine, reserpine, and serotonin in regard to their effects in endotoxemias are obscure, and we find no evidence for the suggestion that the antitoxic activity of chlorpromazine and reserpine can be explained by serotonin increase in the peripheral circulation of treated animals. In fact, after 4 years of intensive research with reserpine, meprobamate, and chlorpromazine in bacterial stress, we are unable to discern any evidence that the antitoxic properties of these three agents are manifested through a single mode of action.

SUMMARY AND CONCLUSIONS

1. Rats and mice were subjected to overwhelming bacterial stress, using purified exotoxins and various lipopolysaccharide endotoxins.

2. In all cases, a single, moderate dose of chlorpromazine administered 1 hour prior to toxin challenge resulted in a prolongation of survival time.

3. Neither hydroxyzine nor methaminodiazepoxide altered survival time significantly in toxin-challenged animals.

4. Doubt is cast on the probability that the antitoxic properties of chlorpromazine are simply a reflection of the release of serotonin into the circulation of treated animals.

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Anticonvulsant Properties of Some N-Substituted Hydantoins

By HAROLD H. WOLF†, EWART A. SWINYARD, and LOUIS S. GOODMAN

The neurotoxic doses, anticonvulsant potencies, and protective indexes of three N-substituted hydantoins were compared with similar values concomitantly determined for phenantoin and diphenylhydantoin. The experimental agents induced overt evidence of neurotoxicity only after doses which were significantly higher than those required for phenantoin and diphenylhydantoin and exhibited anticonvulsant potencies which were generally less than those for the two clinical agents. Of the experimental compounds, 1-methyl-5,5-phenylethylhydantoin appeared most promising. Structure-activity studies indicated that mono-methyl substitution on a nitrogen of the hydantoin ring exerted a more favorable influence on anticonvulsant activity than either di-methyl or mono-ethyl substitution and that optimal activity was obtained when the methyl substitution is on the nitrogen in position 3 of the hydantoin ring.

THE AVAILABILITY of three N-substituted hydantoins offered the opportunity to explore further the structure-activity relation of some phenantoin congeners. Our interest in these compounds was also stimulated by a preliminary clinical report (1) which indicated that one of them is clinically effective in epilepsy. For this reason it seemed desirable to obtain precise experimental data on this agent in order to provide additional information on the correlation between the laboratory assay and clinical efficacy of anticonvulsant drugs.

METHODS

Male albino mice (Carworth Farms, CF No. 1 strain) were used as experimental animals. They were maintained on Rockland mouse diet and allowed free access to food and water except for the short time they were removed from their cages for testing. The following anticonvulsant agents were studied: 1-methyl-5,5-phenylethylhydantoin (No. 1),[1] 1,3-dimethyl-5,5-phenylethylhydantoin (No. 2),[1] 3-ethyl-5,5-phenylethylhydantoin (No. 3),[1] 3-methyl-5,5-phenylethylhydantoin (phenantoin),[2] and diphenylhydantoin.[3] All drugs were given orally as suspensions in 6% acacia solution, except for diphenylhydantoin which was given as the sodium salt in an aqueous solution. The concentration of the drug suspension or solution employed was such that the dose administered always represented 1 ml. per 100 Gm. body weight.

Anticonvulsant potencies (ED_{50}s) were determined by three tests (two electrical and one chemical). The tests based on electrically-induced convulsions measured the ability of a drug to prevent the hindleg tonic-extensor component of maximal electroshock seizures evoked by supramaximal current (MES test; 50 ma. alternating current, 0.2-second stimulus duration, corneal electrodes) and to elevate the threshold for low-frequency electroshock seizures (l.f. EST) induced in mice by unidirectional current delivered at an intensity twice threshold (0.2-millisecond duration, 3-second stimulus duration, six pulses per second). The test based on chemically-induced convulsions measured the ability of a drug to afford complete protection against seizures induced by the subcutaneous injection of pentylenetetrazol (Metrazol; 85 mg./Kg.; s.c. Met. test). A Grass stimulator (model S4B) was used for the l.f. EST test; otherwise the details of the various procedures, the end points employed in mice, and the characteristics of the electroshock apparatus have been described elsewhere (2–4). In addition, the mean neurotoxic dose (TD_{50}) was determined for each drug. The end point for minimal neurotoxicity was muscular incoordination, based on the inability of the animal to remain for 1 minute on a horizontal rod rotating at 6 r.p.m. Each drug was tested at the time of its peak activity as measured by the neurotoxicity test. For the determination of the ED_{50} or TD_{50}, groups of 8 to 12 mice were given various doses of drug until at least three points were established in the range between 0 and 100% seizure protection or minimal neurotoxicity. The results obtained were then plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data the respective ED_{50}, TD_{50}, 95% fiducial limits, and protective index (P.I. = TD_{50}/ED_{50}) were calculated by the method of Litchfield and Wilcoxon (5).

RESULTS

The time of peak effect, neurotoxicity, and anticonvulsant potency of the N-substituted compounds in mice, in comparison with phenantoin and diphenylhydantoin, are shown in Table I. The time of peak effect as measured by the test for minimal neurotoxicity varies from 1 hour for No. 3 to 3

Received April 3, 1961, from the Departments of Pharmacology, University of Utah, College of Pharmacy and College of Medicine, Salt Lake City.
Accepted for publication May 2, 1961.
This investigation was supported by research grants from the National Institute of Neurological Diseases and Blindness (B-381), National Institutes of Health, U. S. Public Health Service, and Sandoz Pharmaceuticals.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
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[1] Kindly supplied by Mr. Harry Althouse, Sandoz Pharmaceuticals. Sandoz code numbers: No. 1 (N-3), No. 2 (N-5), and No. 3 (N-7).
[2] Marketed as Mesantoin by Sandoz.
[3] Marketed as Dilantin by Parke Davis.

TABLE I.—ANTICONVULSANT POTENCY, NEUROTOXICITY, AND PROTECTIVE INDEX (P.I.) OF SOME N-SUBSTITUTED HYDANTOINS AND DIPHENYLHYDANTOIN IN MICE[a]

Drug	Time Peak Effect, min.[b]	TD_{50}	Assay: MES ED_{50}	MES P.I.	l.f. EST ED_{50}	l.f. EST P.I.	s.c. Met. ED_{50}	s.c. Met. P.I.
No. 1	180	292	102	2.86	75	3.89	75	3.89
		(274–311)	(90–115)	(2.60–3.15)	(63–89)	(3.22–4.71)	(60–94)	(3.09–4.90)
No. 2	150	275	110	2.50	100	2.75	83	3.31
		(229–330)	(101–120)	(2.05–3.05)	(86–116)	(2.18–3.47)	(63–110)	(2.40–4.57)
No. 3	60	442	230	1.92	175	2.53	265	1.67
		(402–486)	(172–308)	(1.40–2.63)	(141–217)	(1.96–3.26)	(215–326)	(1.33–2.10)
Phenantoin	90	200	46	4.35	65	3.08	40	5.00
		(167–240)	(39–53)	(3.45–5.48)	(49–87)	(2.20–4.31)	(34–48)	(3.91–6.40)
Diphenyl-hydantoin	180	110	16	6.88	61	1.80	[c]	[c]
		(88–138)	(13–19)	(5.13–9.22)	(41–92)	(1.14–2.84)		

[a] Values in parentheses are 95% fiducial limits; ED_{50}s in mg./Kg. [b] By neurotoxicity test. [c] Not effective by this test.

hours for No. 1 and diphenylhydantoin. Phenantoin and No. 2 exhibit peak activity at $1^1/_2$ and $2^1/_2$ hours, respectively. The TD_{50}s range from 110 mg./Kg. for diphenylhydantoin to 442 mg./Kg. for No. 3. Phenantoin, No. 2, and No. 1 induce overt symptoms of neurotoxicity in 50% of the animals after doses of 200, 275, and 292 mg./Kg., respectively.

With regard to anticonvulsant potency, Table I shows that, except for No. 1 by the l.f. EST test and for diphenylhydantoin by the s.c. Met. test, the N-substituted hydantoins are considerably less potent than either phenantoin or diphenylhydantoin. This is clearly established by examining the potency ratios obtained by dividing the ED_{50} for phenantoin by the ED_{50} for No. 1. Thus, the potency ratio is 0.45 by the MES test and 0.53 by the s.c. Met. test. On the other hand, there is no significant difference between the potency of No. 1 and the two clinically established hydantoins by the l.f. EST test. Furthermore, No. 1 is effective by the s.c. Met. test in a dose of 75 mg./Kg., whereas diphenylhydantoin is ineffective by this test.

Table I also lists the protective indexes (P.I.) of the five compounds as derived from their TD_{50}s and ED_{50}s by the various tests. It may be seen from the table that No. 1 exhibits the highest P.I. of the experimental N-substituted compounds by all three tests and that these P.I.s are significantly higher than those for No. 3. However, the difference between the P.I.s for No. 1 and No. 2 is significant only in the case of the l.f. EST test. Except for No. 1 by the l.f. EST test, the P.I.s for the N-substituted compounds are all less than those for phenantoin. Thus, the P.I.s for No. 1 and phenantoin by the MES test are 2.86 and 4.35, respectively, and by the s.c. Met. test 3.89 and 5.00, respectively. Although the N-substituted compounds all have P.I.s less than diphenylhydantoin by the MES test, they all exhibit P.I.s greater than those for diphenylhydantoin by the l.f. EST and s.c. Met. tests. In addition, No. 1 has the highest P.I. of any of the five compounds examined when compared on the basis of the l.f. EST test; however, the difference between the P.I.s for No. 1 and phenantoin by this test is not significant.

DISCUSSION

The data presented indicate that, except for diphenylhydantoin as measured by the s.c. Met. test, the profiles of anticonvulsant activity of the N-substituted compounds are qualitatively similar to those of phenantoin and diphenylhydantoin. Thus, all drugs tested have the ability to abolish the hindleg tonic-extensor component of maximal electroshock seizures and to elevate the threshold for l.f. EST and s.c. Met. in nontoxic doses. The ineffectiveness of diphenylhydantoin by the s.c. Met. test is in agreement with other reports from our laboratories (3, 6, 7).

The sequence of neurotoxic manifestations which follow the oral administration of the experimental N-substituted hydantoins and phenantoin is very similar, but there is a threefold difference in the time for maximum neurotoxic effect (compare No. 1 and No. 3, Table I) and a somewhat more than twofold difference in the dosage required to produce minimal neurotoxicity (compare No. 3 and phenantoin, Table I). The ratios of the TD_{50}s (phenantoin or diphenylhydantoin/N-compounds) indicate that the N-substituted compounds are only 45 to 73% as toxic as phenantoin and 25 to 40% as toxic as diphenylhydantoin.

With regard to anticonvulsant activity, No. 1 is more effective in elevating the threshold for electrically- and chemically-induced seizures than in modifying the pattern of seizures induced by supramaximal electroshock. Conversely, diphenylhydantoin is more effective in modifying the pattern of maximal electroshock seizures than in elevating the threshold for electrically- and chemically-induced seizures. On the other hand, No. 2, No. 3, and phenantoin do not exhibit significant selectivity of action when compared for ability to modify pattern and to elevate seizure threshold.

The two methyl-substituted hydantoins (No. 1 and No. 2) are significantly more potent by all three tests and significantly more toxic than the ethyl-substituted hydantoin (No. 3). Except for No. 1 by the l.f. EST test, there is no significant difference in the anticonvulsant potency or the neurotoxicity of No. 1 and No. 2. A comparison of phenantoin and No. 2 reveals that mono-methyl substitution (phenantoin) has more influence on anticonvulsant potency than di-methyl substitution (No. 2). Comparison of the anticonvulsant potencies of the two mono-methyl compounds (No. 1 and phenantoin) indicates that methyl substitution in position 3 induces approximately a twofold increase in the anti-MES and anti-s.c. Met. activity, but has no significant effect on the l.f. EST. These observations suggest that N-methyl substitution increases anticonvulsant activity more than does N-

ethyl substitution, and that for optimal activity the N-methyl substitution on the hydantoin ring should be in position 3, as in phenantoin.

An evaluation of the P.I.s derived from the experimental data reveals that No. 1 has the highest value of the three experimental drugs tested. Comparison of the P.I.s of No. 1 and those of the two clinical compounds indicates that, except for diphenylhydantoin by the s.c. Met. test, all three agents exhibit satisfactory P.I.s by all three tests. The difference between the P.I.s for No. 1 and phenantoin by the l.f. EST and s.c. Met. tests is not significant.

A preliminary clinical report (1) and unpublished observations communicated to us by Dr. Leonard W. Jarcho, Division of Neurology, University of Utah, suggest that No. 1 is effective in some patients with grand mal epilepsy resistant to other anticonvulsant therapy and, in contrast to phenantoin and diphenylhydantoin, in some patients with petit mal. Observed side effects are said not to be serious and, to date, gum hypertrophy has not been reported. Should more extensive clinical studies unequivocally establish that No. 1 is effective in patients not responding to conventional antiepileptic therapy and does indeed possess a broader spectrum of activity and lower incidence of toxicity than do the related hydantoins, it would represent a significant addition to the drugs currently available for the treatment of epilepsy.

SUMMARY

The anticonvulsant potencies (ED_{50}s) of three experimental N-substituted hydantoins, 1-methyl-5,5-phenylethylhydantoin (No. 1), 1,3-dimethyl-5,5-phenylethylhydantoin (No. 2), and 3-ethyl-5,5-phenylethylhydantoin (No. 3), and of phenantoin and diphenylhydantoin were determined in mice by the following three tests: maximal electroshock seizure pattern (MES) test, low-frequency electroshock seizure threshold (l.f. EST) test, and pentylenetetrazol seizure threshold (s.c. Met.) test. In addition, the dose of each drug which produced minimal evidence of overt neurotoxicity in 50% of animals (TD_{50}) was determined and protective indexes (TD_{50}/ED_{50}) were calculated. On the basis of the results obtained the following conclusions appear justified:

1. The TD_{50}s for the three experimental drugs are significantly higher than those for phenantoin and diphenylhydantoin.

2. The N-substituted compounds exhibit activity by all three tests; but, except for diphenylhydantoin by the s.c. Met. test and No. 2 by the l.f. EST tests, they are less potent than the two clinically employed agents. Number 1 is more effective in elevating seizure threshold than in modifying maximal seizure pattern, whereas diphenylhydantoin is more effective in modifying maximal seizure pattern than in elevating seizure threshold. Number 2, No. 3, and phenantoin exhibit no striking selectivity of action.

3. On the basis of P.I.s, No. 1 appears to be the most promising of the three experimental drugs tested. The P.I. for No. 1 by the MES test is significantly lower than that for phenantoin or diphenylhydantoin, but by the l.f. EST and s.c. Met. tests its P.I.s are significantly higher than those for diphenylhydantoin and not significantly different from those for phenantoin.

4. Mono-methyl substitution (No. 1 and phenantoin) exerts a more favorable influence on anticonvulsant activity than does either dimethyl (No. 2) or mono-ethyl (No. 3) substitution. Except for activity as measured by the l.f. EST test, the drug with methyl substitution in the 3 position (phenantoin) is more neurotoxic and exhibits significantly more anticonvulsant activity than the compound with the methyl group in position 1 (No. 1).

5. No. 1 appears worthy of more definitive clinical trial.

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Evaluative Procedures for Film-Forming Materials Used in Pharmaceutical Applications

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The current interest in films for use in many pharmaceutical applications has motivated a study in which meaningful testing procedures have been developed or adapted for evaluating such films. The testing procedures studied were found to be rapid, efficient, reproducible, and valid for the evaluation of the pertinent properties of several different series of film-forming materials. The studies were conducted on the free films which were cast from these materials. Data gathered on solubility, moisture absorption, water vapor transmission rates, and the effects of temperature, humidity, and light on the free films indicate that the testing methods may be used to predict the effects of these variables on the performance of the films when they are utilized in a dosage form.

THE POTENTIAL utilization of films as specialized coatings on medications, as vehicles for medicaments, or as packaging agents has prompted several studies within recent years which evaluated various film-forming materials for these applications.

A study of polyvinylpyrrolidone (1) as a film former established a simple and rapid method for coating tablets. Similar studies were made using polyethylene glycol (2), hydroxyethylcellulose and sodium carboxymethylcellulose (3), and zein (4) as the coating materials. Several patents have been issued on film-coating materials such as silicones (5), sodium carboxymethylcellulose (6), and cellulose acetate phthalate (7). Other investigations have utilized films as moisture-protective barriers on such medicaments as ascorbic acid (8) and sodium *p*-aminosalicylate (9). Films of various compositions have been studied as enteric coatings (10) and as vehicles for medicaments (11).

All of the reported studies indicated that when films were investigated for use as specialized coatings on dosage forms, the film-forming agents were evaluated after being applied to the dosage form. This procedure often gives rise to variables which are not introduced by the film itself and may lead to erroneous or misleading results which may serve to deter an investigator from further study. In addition, many important properties of the film itself are evaluated only with difficulty after it was been applied to a dosage form. The addition of special modifiers to the film or the combination of two or more film-forming materials in one formula often results in altering the properties of the film. It is therefore desirable to assess the effect of such modifications before applying the film to a dosage form. In this manner, the properties of the free film could be evaluated without being influenced by the nature of the dosage form or the coating technique.

It was the purpose of this study to utilize free films as a means of establishing rapid screening procedures which would evaluate the critical properties of films *per se*. The objectives were (*a*) the selection of simple methods for accurately producing uniform test films, and (*b*) the adaptation of methods for rapidly evaluating these films.

EXPERIMENTAL

The five major film characteristics which were selected as being most critical in pharmaceutical applications are: (*a*) solubility, (*b*) moisture absorption, (*c*) water vapor transmission, (*d*) reaction to temperature, and (*e*) reaction to light. Other characteristics which were not included in this study are adhesiveness, tensile strength, hardness, and oxygen transmission. These might be of importance in special cases.

Apparatus

For Film Casting.—The Gardner Ultra Applicator[1] was selected over other types of film-forming devices since it offered the advantages of simplicity of adjustment and operation in the casting of films. The Ultra Applicator (Fig. 1) is composed of two parts, a block and a blade. The stainless steel rails attached to the block are tapered at a gradient of 10 mils per 2-inch length. A thumb screw holding the blade to the underside of the block permits the

Received April 11, 1961, from Columbia University, College of Pharmacy, New York 23, N. Y.
Accepted for publication October 18, 1961.
Taken in part from a thesis submitted by Harris Goodman in partial fulfillment of the requirements for the degree of Master of Science, Columbia University, College of Pharmacy, June 1960.
Presented to the Section on Pharmacy, meeting of the American Association for the Advancement of Science, New York, N. Y., December 29, 1960.
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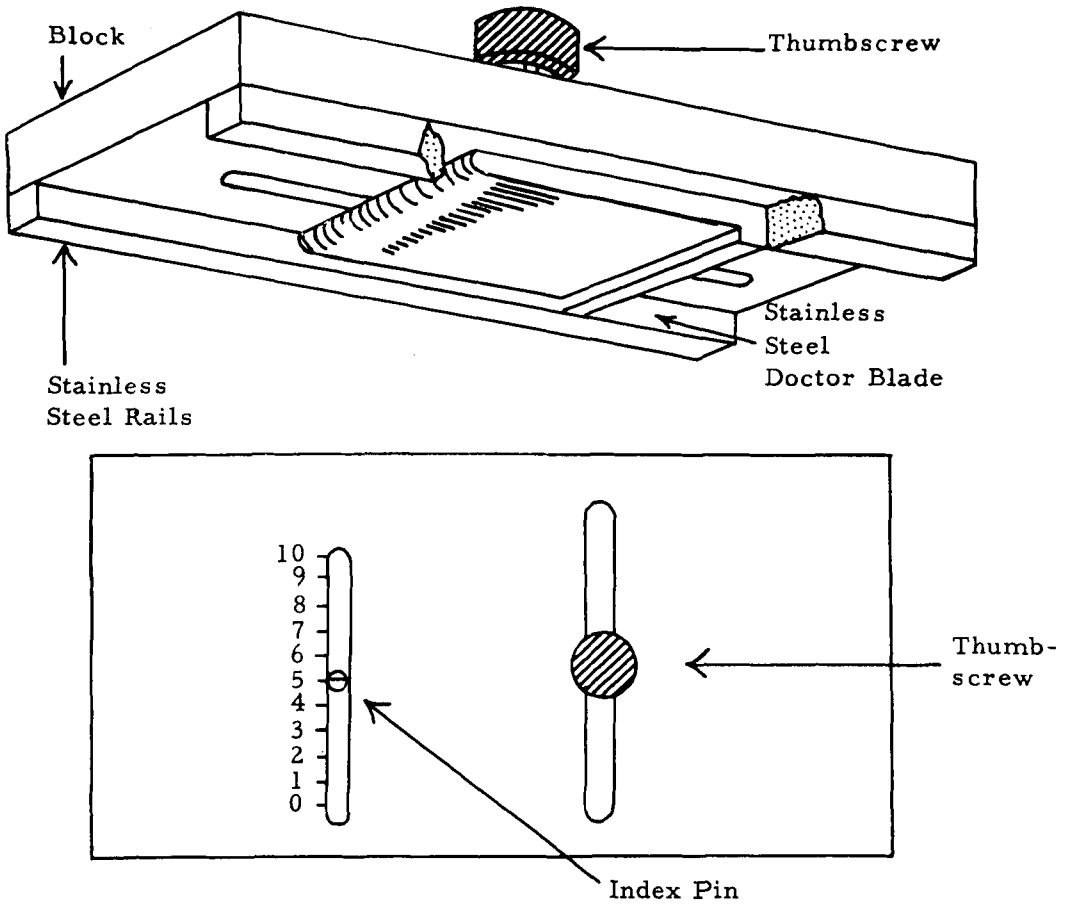


Fig. 1.—Gardner Ultra Applicator.

positioning of the blade along the gradient so that films of any desired wet film thickness may be drawn on a suitable surface. The critical dimensions of the applicator itself are held within 0.0001 inch tolerance which assures optimum accuracy of draw downs over its entire length. Models of the Ultra Applicator are available for producing wet films ranging from 0 to 20 mils in thickness, and from 0.75 to 6 inches in width.

When used, the blade position is adjusted for the desired wet film thickness and the block is placed over a small pool of the solution of the film former on a smooth surface. The entire applicator is then drawn downward over the pool to spread it evenly over the surface. The resulting wet films are then dried and removed for evaluation.

The surface onto which the films are cast also affects the production of uniform films. Highly polished chromium plated stainless steel plates, measuring 8 by 13 inches, were selected for this purpose since it has been shown that film-forming materials drawn over such a surface encounter less resistance in flowing over it than over ordinary steel surfaces (12).

For Film Evaluation.—The dry film thickness was measured by means of an Ames dial comparator, model 2w.[2] This dial comparator was fitted with an indicator capable of accurately recording 0.0001 inch thicknesses. Contact point area and contact pressure was designed to meet A.S.T.M. standards for thickness measurement of plastic film or similar materials. Contact pressure was controlled by use of a 19-oz. dead weight.

Water vapor transmission (W.V.T.) rates of free films were established by using the water vapor permeability cup, No. 305-7, Carson type, cast wax seal.[3] This type of vapor permeability cup meets the specifications of the A.S.T.M. test No. E96-53T, and is available with wax casting template and weighing cover.

Materials

In order to evaluate films which are indicative of a wide range of pharmaceutical applications, this investigation was conducted with the following film forming agents which represent four major groups of films based on solubility. The agents which were used were: (*a*) ethylcellulose, representing water-insoluble films; (*b*) methylcellulose, representing water-soluble films; (*c*) zein, representing varying solubility depending on pH; and (*d*) polyvinylpyrrolidone and ethylcellulose combination, representing slowly soluble films.

[2] B.C. Ames Co., Waltham 54, Mass.

[3] Thwing Albert Instrument Co., Philadelphia 44, Pa.

The combination of polyvinylpyrrolidone and ethylcellulose was chosen because it offered the possibility of evaluating a film which was composed of water-soluble and water-insoluble components, both of which are soluble in a common solvent such as ethanol, and could thus be cast as a single film.

Preliminary work was done to establish the optimum conditions for film casting and drying. Various solvent systems, concentrations of film forming materials, wet film thicknesses, and drying times and temperatures were tried for each of the materials studied.

Procedure

Once a particular formulation was found to be suitable, a bulk solution of sufficient volume was prepared from which all film samples could be produced for evaluation. The following solutions were used in this investigation:

Solution A	
Ethylcellulose, 50 cps.	10% w/v
Alcohol U.S.P.	20% v/v
Toluene	70% v/v

Films of various thicknesses were produced by drying the wet films at 94° for 8 minutes.

Solution B	
Methylcellulose, 400 cps.	5% w/v
Purified water	95% v/v

Films were prepared at a drying temperature of 80° at drying times of 8 to 14 minutes.

Solution C	
Zein	21.60% w/v
Oleic acid	15.90% v/v
Alcohol U.S.P.	56.25% v/v
Purified water	6.25% v/v

Films were produced from this solution after drying the wet films for 6 to 10 minutes at 51°.

Solution D	
Ethylcellulose, 50 cps.	10.2% w/v
Polyvinylpyrrolidone	3.2% w/v
Citroflex a-4	3.5% v/v
Alcohol U.S.P.	83.1% v/v

Films were developed and dried for 4 minutes at 44°.

Oleic acid in solution C, and Citroflex a-4 in solution D were added as plasticizers to eliminate brittleness of films as they dried.

Testing of Films

Solubility of Films.—Film samples were cut to uniform size by using a 1 × 3 inch glass microscope slide as a cutting template. A scalpel was used to cut strips of films of these dimensions and these strips were evaluated initially and again at stated intervals after being exposed to test conditions.

A sufficient number of strips of each different film of various thicknesses was stored in 4-ounce, wide-mouth glass containers with plastic screw caps and maintained at room temperature, 40°, and 60°. Samples of each were withdrawn at 1, 2, 4, 8, and 12-week intervals and were checked for any changes in solubility. In all cases, solubility tests were conducted in both artificial gastric and artificial intestinal fluids. The U.S.P. formulations for these solutions were employed.

The strips of film were immersed completely in 100 ml. of test fluid which was maintained at 37°. The test fluid was constantly kept in motion by means of a magnetic stirrer and the rate of movement was adjusted so as to create and maintain a small vortex. The solubility of the films in the testing solutions was rated visually; the end point being a complete solution of the film. Reproducibility of results was obtained within a very small range of time measured in seconds in several determinations of different samples of the same film. A similar evaluation procedure was used by Jarrell in a study involving the solubility of carboxymethylcellulose films (13).

Moisture Absorption by the Films.—A standardized method for determining water absorption by plastics has been approved by the A.S.T.M. and has been designated as A.S.T.M. test No. D570-59T. This test is designed for all shapes of plastics, including sheets, rods, and disks. A modification of this method was used for the testing of films in this study as follows: Films were prepared and cut into 1 × 3 inch strips. The strips were then conditioned by placing them in an oven at the temperatures and for the drying times which had been used originally in drying the wet films. This step was carried out to insure uniformity of the films within each group before testing. The conditioned sample strips were then suspended by means of fine wire in relative humidity chambers. The wire was made to pierce each strip in such a manner as to permit free access of the moist air to all surfaces of the films. Three chambers were set up to produce relative humidities of 10, 52, and 93%, respectively. The relative humidity in the chamber was controlled by the use of different saturated solutions containing excess solute. The conditioned film specimens were accurately weighed, placed in the relative humidity chambers, and then removed and weighed again at the end of 1, 2, 4, 8 and 12-week intervals. Increase or decrease in weight, changes in physical appearance or in solubility were then observed. Per cent moisture absorption was calculated by means of the following formula

$$\frac{\text{weight of exposed film} - \text{weight of conditioned film}}{\text{weight of conditioned film}} \times 100$$

The percentages calculated by means of this formula were plotted against time to establish the point of maximum absorption for each specimen film.

Moisture absorption data on films must include consideration of the following factors before the results may become applicable: (*a*) dimensions of the specimen, (*b*) conditioning time and temperature, (*c*) exposure procedure used, (*d*) time intervals of exposure and sampling, (*e*) percentage increase in weight, (*f*) observations as to changes in physical appearance of film specimens, and (*g*) effects on solubility.

Water Vapor Transmission.—The method employed to measure W.V.T. was a modification of the A.S.T.M. test No. E96-53T. Procedure E of this test for materials in sheet form was adapted for measurements of W.V.T. at an elevated temperature, with a low humidity on one side of the film and a high humidity on the other side.

The Carson type water vapor permeability dish was employed as the testing apparatus. These aluminum dishes are made with specially designed

flared edges upon which disks of film specimens may be placed and sealed in place with a wax seal. Drierite desiccant, No. 8 mesh, was placed within the dish to a height of within 3 mm. of the film sample being tested. In order to insure perfect sealing of the film disk to the edges of the dish and thus prevent leakage of water vapor around the film, a specially designed template was employed to define the test area and to help in forming the wax seal. The template was carefully placed over the film specimen, which was resting on the edges of the dish, and molten wax was poured into the space between the beveled edge of the template and the outer edge of the dish. When the wax solidified, the template was removed by gently twisting it until it came free. A mixture of 60% microcrystalline wax and 40% paraffin was found to be adequate for sealing the edge of the film to the edge of the dish.

Four specimens of each film were prepared in this manner. The entire assembly was accurately weighed and then placed in a constant humidity chamber at 37.8 ± 1.5° and 91% R.H. ±2%. A control blank apparatus was included in each batch for the purpose of establishing if any significant change could be attributed to the test apparatus itself. No significant change was noted at any time.

The W.V.T. of a sample was calculated in terms of grams per square inch per 24 hours as follows

$$\text{W.V.T.} = \frac{(g)\,(24)}{(t)\quad(a)}$$

where g = weight change in grams, t = time, in hours, during which weight change occurred, and a = area of exposed specimen film in square inches.

Light Stability Test.—Film samples were cut into 1 × 3 inch strips and placed in open Petri dishes. These were positioned in a light stability cabinet[4] recently described by Lachman and his coworkers (14). The cabinet is constructed so that samples may be placed at various distances from the light source so that light intensity to which samples are subjected may be regulated. By placing the samples at a distance of three feet from the light source, they were subjected to a twentyfold increase in intensity over ordinary light. Samples were studied visually for possible changes in appearance at the end of 4 and 6-week intervals.

RESULTS AND DISCUSSION

Effect of Storage on Solubility of Films.—The four groups of films tested offer a wide degree of solubility characteristics. As had been expected, films of methylcellulose were soluble in the artificial digestive test fluids. No solubility changes were observed in these films upon storage at elevated temperatures for 12 weeks. As had equally been anticipated, films of ethylcellulose were completely insoluble in both gastric and intestinal fluids, and the insolubility of the films was not altered after periods of storage at different relative humidities or temperatures. Different film thicknesses also had no effect on the solubility of these films. Specimen films were considered insoluble when they indicated no change after 24 hours immersion in the testing apparatus.

The results of the solubility studies on zein films substantiated the properties of this material as being insoluble in gastric fluids but rapidly soluble in intestinal fluids. The rate of solubility in artificial intestinal fluid was directly proportional to the thickness of the film, ranging from 1.5 seconds for test strips of 0.6 mil thickness to 9 seconds for test strips of 1.5 mil thickness. The solubility rates of the zein films were in no way altered by the storage conditions.

The combination of ethylcellulose and polyvinylpyrrolidone proved to be completely insoluble in both gastric and intestinal fluids. The original anticipation that this combination would yield a slowly soluble film due to contributory characteristics of each component did not materialize. The possibility exists that the ethylcellulose completely dominated the film characteristics by coating the polyvinylpyrrolidone molecules, and that different combinations of these materials might reverse this situation.

Storage Stability of Films.—The effects of elevated temperatures on the physical characteristics of the film other than its solubility should be noted. Films of ethylcellulose and methylcellulose were found to be completely stable under all temperature conditions. There were no physical changes in these films after 12 weeks. Definite physical changes in the zein films were observed. These changes involved a darkening in color and an increased brittleness. In the case of the thinnest film (0.6 mil) the change became apparent at the 40° temperature level at the end of 8 weeks. At the end of the same period, samples of all thicknesses at 60° had become brown in color and brittle. The cause of this discoloration and increased brittleness was traced to the oleic acid plasticizer used in this film. Oleic acid at 80° has been shown to absorb approximately four atoms of oxygen per molecule with a low yield of peroxide and a considerable evolution of water and carbon dioxide (15). It has also been noted that the oxidation reaction is accelerated by heat and light (16). The long term exposure of the film to the elevated temperature might have caused the acceleration of this oxidation of oleic acid producing a discoloration and a brittleness of the film. The use of other plasticizers such as ethyl lactate, propylene glycol, or sodium lactate might eliminate this problem. No reports could be found in the literature to indicate previous knowledge of such a problem with an oleic acid plasticized zein film.

Moisture Absorption by Films.—The method developed for evaluation of the effect of relative humidity on film properties produced results which were uniform and reproducible. In the case of methylcellulose, results indicated that these films absorbed relatively low amounts of moisture, and reached a point of equilibrium at the end of 3 weeks in relative humidities of 10 and 52%. These relative humidities did not produce any apparent physical changes in the films at the end of 12 weeks.

Moisture absorption in the 93% R.H. chamber, however, was so great that the films became soft and gummy within 4 weeks, the point at which maximum absorption was reached. Figure 2 indicates the percentage of moisture absorption for methylcellulose films of 0.5 mil thickness.

[4] The authors are indebted to Ciba Pharmaceutical Products Inc., Summit, N. J., for making available the light stability cabinet used in this study.

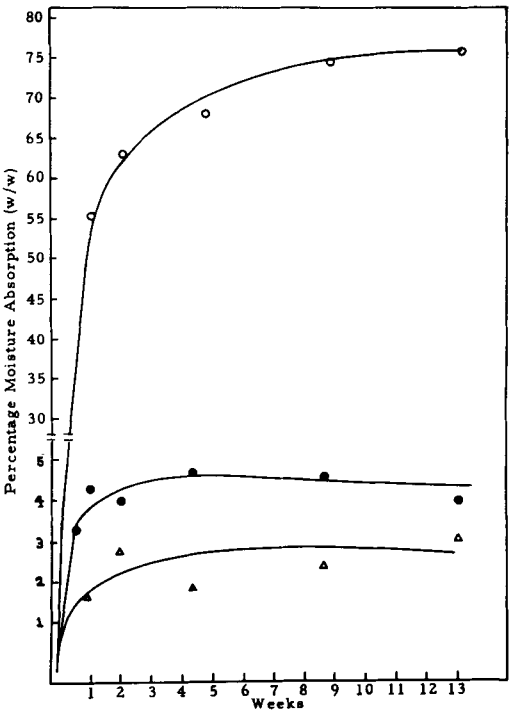


Fig. 2.—Moisture absorption of methylcellulose film of 0.5 mil thickness. O, 93% R.H.; ●, 52% R.H.; △, 10% R.H., all at 37.8°.

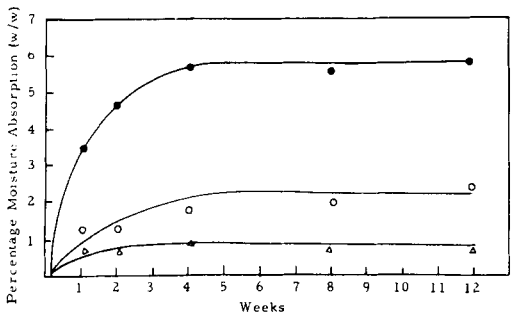


Fig. 3.—Moisture absorption of ethylcellulose film of 1 mil thickness. O, 93% R.H.; ●, 52% R.H.; △, 10% R.H., all at 37.8°.

The amount of moisture absorption by the ethylcellulose films reached equilibrium in 3 to 4 weeks (Fig. 3). It was discovered that the percentage of absorbed moisture was not directly proportional to relative humidity. Regardless of thickness, the films consistently absorbed a higher percentage of moisture at 52% R.H. than they did at 93% R.H. This odd phenomenon was not reported anywhere in the literature and no feasible explanation of this can be offered at this time. Relative humidities at the different levels had no visible effect on the physical properties of the ethylcellulose films.

The zein films showed moisture absorption patterns similar to those of methylcellulose. In relative humidities of 10 and 52%, the moisture absorption was 1.5% or less; equilibrium being established within 3 weeks. Films stored at 93% R.H. were softened considerably by the end of the first week and in some instances the test strips became completely deformed.

The value of studying films produced by a combination of film-forming agents was demonstrated effectively by the moisture absorption rates of the ethylcellulose-polyvinylpyrrolidone films. It has been shown that the moisture content of polyvinylpyrrolidone films is directly related to relative humidity; with the equilibrium percentage of moisture being about one-third of the relative humidity (17). Thus, it could be calculated that if a polyvinylpyrrolidone film was exposed to a relative humidity of 90%, the moisture content at equilibrium would approach 30%. Likewise, at relative humidities of 10 and 52%, the moisture absorption of a polyvinylpyrrolidone film would reach equilibrium at about 3 and 17%, respectively.

Figure 4 indicates that the film composed of a combination of ethylcellulose and polyvinylpyrrolidone absorbed only 0.6% at the 10% R.H. level, and between 3 and 5% moisture at the 52% level. It therefore appears that at the lower levels of relative humidity the ethylcellulose content of the film exercised a major role in the film properties achieved. However, in the 93% R.H. chamber, the same film continued to absorb moisture, and at the end of 12 weeks had absorbed as much as 12%. In this instance it is quite probable that the inherent hygroscopic properties of the polyvinylpyrrolidone overshadowed the effect of the ethylcellulose content. This phenomenon was not observed in ethylcellulose-polyvinylpyrrolidone films of 1.0 mil thickness, in which instance a maximum of 7% absorption of moisture in the 93% R.H. chamber was recorded and this level remained constant after 3 weeks.

Water Vapor Transmission.—Ethylcellulose films showed a W.V.T. rate which varied inversely with the thickness of the film. Figure 5 indicates that the W.V.T. decreased almost linearly with an increased thickness of film. Figure 6 demonstrates that methylcellulose films exhibited W.V.T. charac-

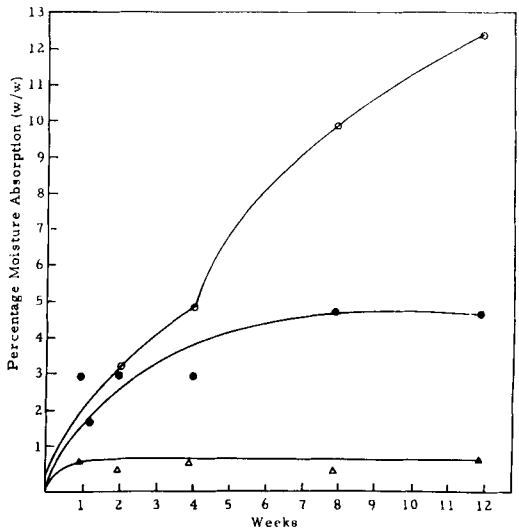


Fig. 4.—Moisture absorption of ethylcellulose-polyvinylpyrrolidone film of 1.5 mil thickness. O, 93% R.H.; ●, 52% R.H.; △, 10% R.H., all at 37.8°.

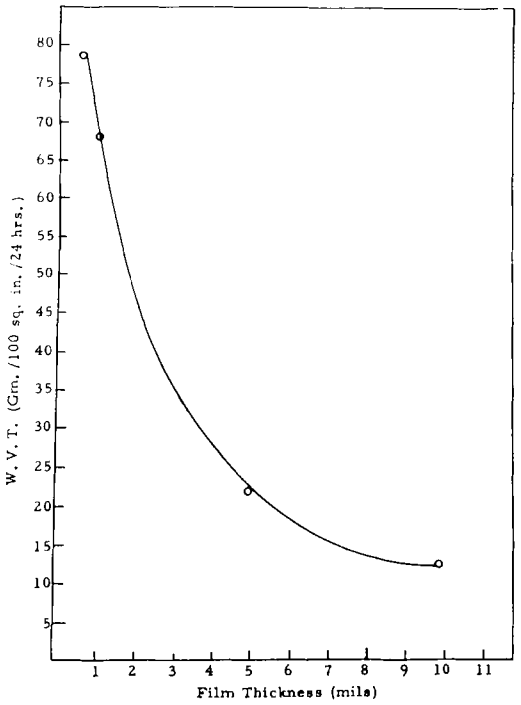


Fig. 5.—Water vapor transmission of ethylcellulose films, 91% R.H. at 37.8°.

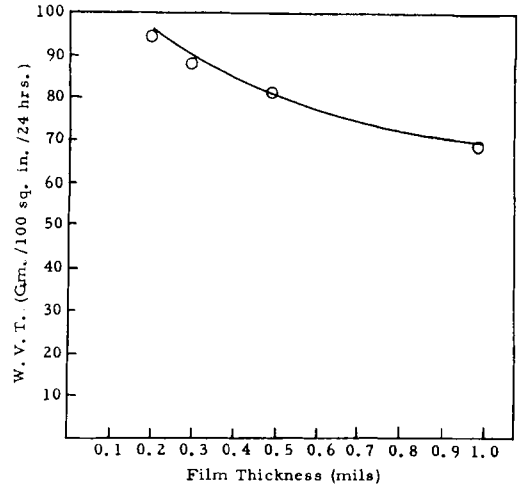


Fig. 6.—Water vapor transmission of methylcellulose films, 91% R.H. at 37.8°.

teristics which were very similar to those of ethylcellulose films, even though the former material is completely soluble in water while the latter is insoluble.

Zein films (Fig. 7) manifested the lowest W.V.T. rates of all the tested films. These films should, therefore, offer good protection qualities against moisture when applied to dosage forms. It has been shown that the major factors controlling the W.V.T. of a film are: chemical structure, polarity, symmetry, and the size of the polymers or molecules making up the film (13, 18). A recent study (19) has demonstrated that the films containing more polar groupings permit larger amounts of moisture vapor to pass through them. The low W.V.T. of the zein films, therefore, may be attributed to the large number of nonpolar side chains making up the polymer units.

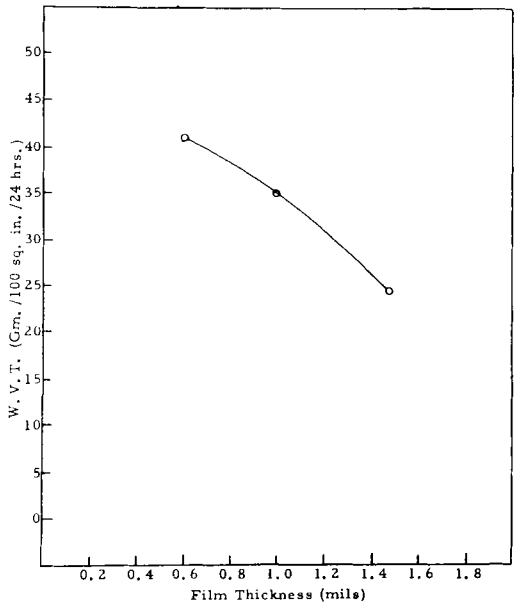


Fig. 7.—Water vapor transmission of zein films, 91% R.H. at 37.8°.

Light Stability of Films.—Samples of all films exposed for a period of 6 weeks in the light stability cabinet indicated complete physical stability with the exception of zein, which underwent a change from an initial light tan color to almost colorless in this period of time. The brittleness of the film was increased at the same time and may have been caused by the oxidation of the oleic acid plasticizer as previously noted in the effects of high temperatures on the same film. The discoloration of the film was probably due to a bleaching of the color constituents of zein. These observations suggest the necessity of the use of other plasticizers, and the addition of ultraviolet light-absorbing agents in those instances where bleaching of films is undesirable.

SUMMARY

1. Meaningful testing procedures have been developed or adapted for the rapid evaluation of film-forming agents for pharmaceutical use.

2. The testing procedures developed were used to evaluate several free films, and the results obtained either substantiated expected results or indicated properties which might not have been revealed in an applied film.

3. Data on the physical properties observed as a result of the evaluation of free films not only indicate the validity of the testing procedures but also point out the need for further study of free films in contrast to the current methods of evaluation of applied films.

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Notes

Toxicity and Central Depressant Activity of D-1-Phenyl-2-ureidopropane

By ELVEN K. BAUMAN and ALBERT R. HASKELL†

D-1-Phenyl-2-ureidopropane was prepared by the method of Toritaku and alternatively, by reacting 1-phenyl-2-aminopropane directly with urea. The LD_{50} of this ureide was found to be 840 mg./Kg. The compound was found to act as a central nervous system (CNS) depressant rather than as a sustained action CNS stimulant, as suggested by Toritaku. Comparison of the structural formula of 1-phenyl-2-ureidopropane with that of the barbiturates and hydantoins supports the experimental evidence that this compound acts as a barbiturate-like central nervous system depressant.

THE PREPARATION of sustained release amphetamine compositions has depended principally upon three approaches: (*a*) the preparation of beads of active ingredient coated with shellac, wax, or other insoluble substance, (*b*) the formation of a complex between amphetamine and an ion exchange resin, and (*c*) the formation of insoluble salts of amphetamine, such as the tannate, which are then slowly converted to soluble amphetamine salts in the acid stomach.

Our attention has been directed toward the search for new amphetamine compounds which would hydrolyze to give sustained amphetamine action or which, in themselves, would provide prolonged stimulation of the central nervous system.

Toritaku (1) has indicated that the urea derivative of amphetamine hydrolyzes *in vivo* to give a sustained amphetamine action. Toritaku's patent described the preparation of the compound but reported no biological tests to demonstrate the amphetamine action. This study was therefore initiated to determine the toxicity and duration of action of D-1-phenyl-2-ureidopropane.

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Accepted for publication April 21, 1961.
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EXPERIMENTAL

Synthesis.—D-1-Phenyl-2-ureidopropane was prepared from D-1-phenyl-2-aminopropane sulfate and potassium cyanate as described by Toritaku (Fig. 1).

D-1-Phenyl-2-aminopropane sulfate, 60 Gm. and potassium cyanate, 30 Gm., were placed in 350 ml. distilled water and boiled for 30 minutes. After three recrystallizations from water, white needles were obtained which melted at 138.5 to 139°, which agrees with previously reported data (2, 3). The actual yield from the reaction was 55 Gm. or 90 % of the theoretical yield, 58 Gm.

D-1-Phenyl-2-ureidopropane was also prepared by the reaction of D-1-phenyl-2-aminopropane with urea (Fig. 2.). This method was abandoned because of low yield.

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Results.—The data from the intraperitoneal

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Results.—The data from the intraperitoneal

TABLE I.—INTRAPERITONEAL LD_{50} EVALUATION OF D-1-PHENYL-2-UREIDOPROPANE

Dose, mg./Kg.	Final Dosage Levels and Mortality Ratios[a] Time, hr.										
	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	24
700	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
800	0/6	0/6	0/6	1/6	1/6	1/6	1/6	1/6	2/6	2/6	2/6
900	0/6	0/6	1/6	2/6	3/6	4/6	4/6	4/6	4/6	4/6	4/6

[a] The figures in this table indicate the ratio between the number of animals which succumbed and the number of animals injected.

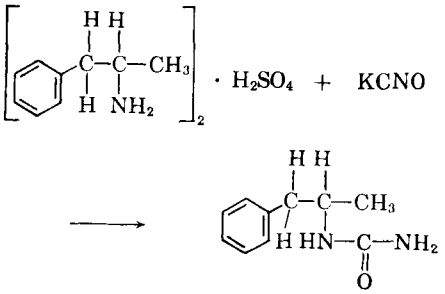


Fig. 1.—Preparation of 1-phenyl-2-ureidopropane from amphetamine sulfate and potassium cyanate.

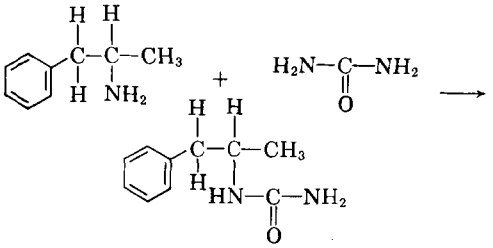
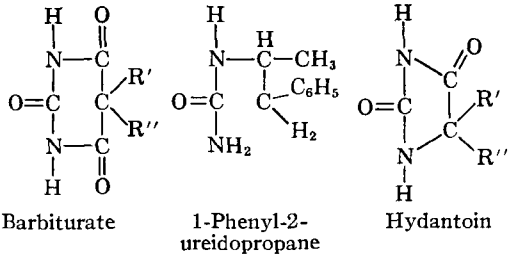


Fig. 2.—Preparation of 1-phenyl-2-ureidopropane from amphetamine and urea.

LD_{50} determination are shown in Table I. The intraperitoneal LD_{50} for 200-Gm. rats appears to be approximately 840 mg./Kg. During this procedure no convulsions or other symptoms of central nervous system stimulation were observed.

When 200-Gm. rats were subjected to an oral dose of this compound equal to two times the intraperitoneal LD_{50} dose the onset of drug activity occurred in approximately 60 minutes and was indicated by general lethargy, loss of irritability, and lack of coordination when walking. Two hours after the administration no voluntary movement of the test animals was observed. At this point shallow breathing was apparent and pulse rates were found to be about one-half of normal. Two of the 12 rats had lost the righting reflex but continued to respond to the tail dip test using water at 160°F. Recovery, indicated by the return of coordinated



Barbiturate 1-Phenyl-2-ureidopropane Hydantoin

Fig. 3.—The structure of 1-phenyl-2-ureidopropane and two typical CNS depressant nuclei.

movement and response to noise, occurred between 6 and 8 hours after administration. All animals survived. It was again observed that no convulsions occurred in the test animals but rather the signs and symptoms of central nervous system depression were apparent.

DISCUSSION

The striking feature of the animal experiment was the absence of any reaction similar to that in rats treated with 1-phenyl-2-aminopropane. In all cases the test animals gave the appearance of having been treated with a central nervous system depressant such as a barbiturate. For example, of 12 animals treated orally, 10 had a marked loss of coordination and the remaining 2 were under light anesthesia, having lost the righting reflex but still responding to pain.

An examination of the structural formula of the compound in question provides an explanation for the observed experimental facts. In Fig. 3 are shown the basic structural components of 1-phenyl-2-ureidopropane, of the barbiturate nucleus, and the hydantoin nucleus. Examination of Fig. 3 will show that the basic components of a central nervous system depressant are present in 1-phenyl-2-ureidopropane and therefore that central nervous system depression from this compound should not have been unexpected.

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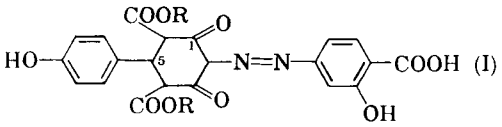
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Coupling of Diazotized *p*-Aminosalicylic Acid (PAS) with 5-(*p*-Hydroxyphenyl)-cyclohexanedione-1,3 and Related Compounds

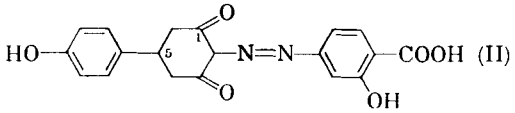
By PHILLIPOS E. PAPADAKIS and S. C. PIRRUCCELLO

The syntheses of five derivatives of PAS are described. The compounds were prepared for study as agents having possible increased antimetabolite and/or antimicrobial properties.

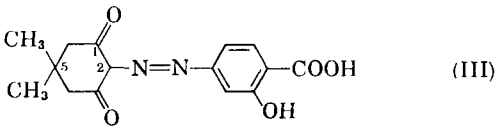
IT IS KNOWN that many azo compounds are useful as dyes, diagnostic agents, indicators, staining agents for bacteria, etc. Some azo compounds depending on the radicals and functional groups involved in their structures have important physiological properties. The fact that PAS has been used with or without other drugs in the chemotherapy of tuberculosis prompted these authors to do this work, namely the synthesis of compounds (I) to (V). This report is an extension of previous work (1) and deals with the coupling of diazotized *p*-aminosalicylic acid with each of the following compounds: (*a*) 5-(*p*-hydroxyphenyl) 4,6-dicarbethoxycyclohexanedione-1,3; (*b*) 5-(*p*-hydroxyphenyl)-cyclohexanedione-1,3; (*c*) 5,5-dimethylcyclohexanedione-1,3; (*d*) 5-(2′, 3′, 4′-trimethoxyphenyl) 4,6-dicarbethoxycyclohexanedione-1,3; (*e*) trimethoxybenzene. The coupled products are represented by the formulas (I) to (V), respectively.



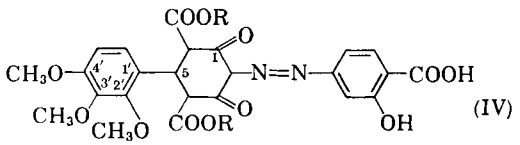
(I)



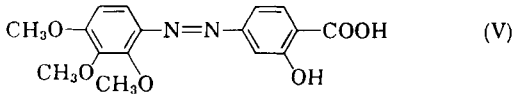
(II)



(III)

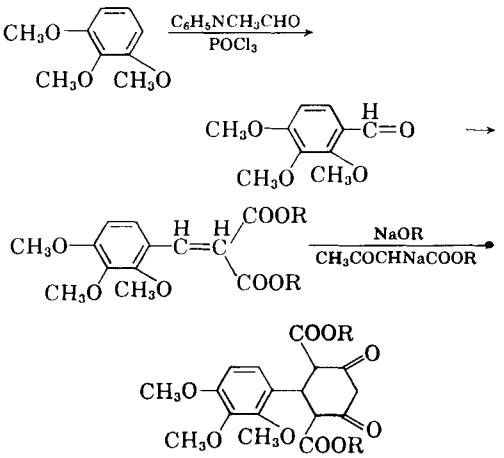


(IV)



(V)

The general methods of the preparation of compounds (*a*) and (*b*) have been previously described (2, 3). The sequence of synthesis for the preparation of compound (*d*) which is a new compound is as follows



The methods used for some of the above syntheses have been described (2, 4).

EXPERIMENTAL

In the diazotization process each of the reagents (*a*) to (*d*) was dissolved in dilute sodium hydroxide and the solution cooled to 0°. In another container an equivalent amount of the sodium salt of *p*-aminosalicylic acid was dissolved in distilled water and to this solution hydrochloric acid was added (calculated to react with: the sodium salt of PAS, the amino group of PAS, and the sodium nitrate). The calculated amount of sodium nitrite was added, a little at a time with stirring. The diazonium salt thus prepared was mixed with the sodium derivative of the respective cyclic dione. The mixture was stirred and then allowed to stand at 3° for 1 hour. Then the mixture was made acid to Congo red paper. A red-brown precipitate was formed which was filtered and washed with distilled water. Products (I) and (III) were recrystallized from 75% alcohol. Products (IV) and (V) were dissolved in ether and reprecipitated with petroleum ether. For the formation of (V) the trimethoxybenzene was dissolved in the minimum quantity of dioxane at 10° and added, with stirring, to the diazonium salt of PAS at 0°. Sodium hydroxide was added to take care of the hydrochloric acid. After stirring for 1 hour at 4° the mixture was made acid to Congo red paper, a black-red precipitate was formed which was filtered, dissolved in ether, and reprecipitated with petroleum ether. The analyses of the compounds following are given in Table I: I, N-2-[5-(*p*-hydroxyphenyl)-4,6-dicarbethoxy-cyclohexanedione-1,3]-azo-N-*p*-salicylic acid; II, N-2-[5-(*p*-hydroxyphenyl)-cyclohexanedione-1,3]-azo-N-(*p*-salicylic acid); III, N-2-[5,5-dimethyl-cyclohexanedione-1,3]-azo-N-(*p*-salicylic acid); IV, N-2-[5-(2′, 3′, 4′-trimethoxyphenyl)-4,6-dicarbethoxy-cyclohexanedione-1,3]-azo-N-(*p*-salicylic acid); V, N-1-(2,3,4-trimethoxybenzene)-azo-N-(*p*-salicylic acid).

Received February 6, 1961, from the School of Pharmacy, Creighton University, Omaha, Nebraska.
Accepted for publication March 9, 1961.

TABLE I.—ANALYSIS OF COMPOUNDS

No.	M.P., °C.	Empirical Formulas	Analysis: Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N
I	...	$C_{26}H_{24}N_2O_{10}\cdot 2H_2O$	54.74	5.11	5.11	54.91	5.58	5.11 5.28
II	d. 186	$C_{19}H_{10}O_6N_2\cdot{}^1/_2H_2O$	60.48	4.61	...	60.78	4.88	...
III	d. 235	$C_{15}H_{16}N_2O_5\cdot H_2O$	55.89	4.96	...	55.87	4.78	...
IV	232	$C_{28}H_{30}N_2O_{12}\cdot H_2O$	55.62	5.29	...	55.66	5.37	...
V	...	$C_{16}H_{16}N_2O_6\cdot H_2O$	...	...	8.00	...	...	7.89

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New Alkaline-Stable Species for Selected Members of the Tetracycline Family

Sir:

We have recently observed a new modification of selected members of the tetracycline family which shows greatly enhanced stability under alkaline conditions. This derivative is a complex having the following general formula: (tetracycline - group - antibiotic) - $(aluminum)_a$-$(calcium)_b$-(gluconic acid)$_c$ where a, b, and c are the molar ratios of the respective constituents. Although the molar ratios a, b, and c can vary over a wide range in this derivative, greatly enhanced alkaline stability occurs only when the molar ratios of aluminum and calcium are three or greater. The molar ratio of gluconic acid does not appear to contribute significantly to the alkaline stability, but appears to play an important role in solubilizing the derivative.

In measuring the alkaline stability of derivatives in this series, extensive use has been made of half-life determinations, as reported by McCormick, *et al.* (1). Half-life values have been determined in 0.1 *N* sodium hydroxide at 90–100° by following the time change in (*a*) ultraviolet absorbance at 365 mμ and (*b*) the microbiological activity of the antibiotic.[1] Table I *demonstrates* the enhanced alkaline stability of selected members of the tetracycline family in this series of derivatives.

[1] Microbiological assays were performed under the direction of Dr. J. J. Corbett, Biological Assay Development Laboratory.

TABLE I.—RELATIVE ALKALINE STABILITY OF VARIOUS DERIVATIVES OF 6-DEMETHYLCHLORTETRACYCLINE (DMCTC),[a] CHLORTETRACYCLINE (CTC),[a] 6-DEMETHYLTETRACYCLINE (DMTC), TETRACYCLINE (TC),[a] AND 6-DEOXY-6-DEMETHYLTETRACYCLINE (DODMTC)

	Half-Life, min., in 0.1 *N* NaOH at 90–100°: Spectrophotometric Determination	Half-Life, min., in 0.1 *N* NaOH at 90–100°: Microbiological Determination
DMCTC·HCl	40	60
DMCTC-aluminum-gluconate (1:4:6.6)	95	..
DMCTC-aluminum-calcium-gluconate (1:4:1:12)	1,120	..
DMCTC-aluminum-calcium-gluconate (1:4:2:12)	3,000	..
DMCTC-aluminum-calcium-gluconate (1:4:3:12)	Very long[b]	..
DMCTC-aluminum-calcium-gluconate (1:4:5:12)	Very long[b]	Very long[b]
CTC·HCl	4	..
CTC-aluminum-calcium-gluconate (1:4:5:12)	12	..
CTC-aluminum-calcium-gluconate (1:4:7:12)	26	..
DMTC·HCl	32	..
DMTC-aluminum-calcium-gluconate (1:4:5:12)	Very long[b]	..
TC·HCl	7	..
TC-aluminum-calcium-gluconate (1:4:5:12)	Very long[b]	..
DODMTC·HCl	200	..
DODMTC-aluminum-calcium-gluconate (1:4:5:12)	Very long[b]	..

[a] The trademarks of the American Cyanamid Co. for 6-demethylchlortetracycline, chlortetracycline, and tetracycline are Declomycin, Aureomycin, and Achromycin, respectively.
[b] No change observed after 4 to 6 hours in 0.1 *N* sodium hydroxide at 90–100°.

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DMTC-aluminum-calcium-gluconate (1:4:5:12)	Very long[b]	..
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The various members of the tetracycline family in the table above demonstrate the effects of methylation, chlorination, and deoxygenation of the tetracycline ring system on the ability of complex formation to slow the rate of alkaline degradation. Chlortetracycline is unusual in that repeated alkaline stability studies with complexes described with this antibiotic have failed to demonstrate greatly enhanced alkaline stability.

The resistance of certain of these derivatives to attack by alkali enables them to be prepared as stable solutions when the solution is sufficiently alkaline to avoid C·4 epimerization occurring in the pH range of 2–6 (2) and anhydro formation occurring under very acidic conditions.

Those derivatives showing enhanced alkaline stability experience little or no change in their susceptibilities toward acid degradation and toward C·4 epimerization in comparison to the uncomplexed antibiotic. The lack of significant change toward acid degradation is of particular interest because both alkaline degradation and acid degradation involve chemical alteration of the C·6 hydroxyl, together with other changes in ring C in the antibiotic. The fact that complexing can produce great changes in the alkaline reactivity of the C·6 hydroxyl and little change in the acid reactivity of the C·6 hydroxyl is an important clue to the mechanism of alkaline stability enhancement via complex formation. When 6-deoxy-6-demethyltetracycline is used as the antibiotic in this derivative, greatly enhanced stability toward acid (3) and alkali is observed.

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Received November 13, 1961.
Accepted for publication November 21, 1961.

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The various members of the tetracycline family in the table above demonstrate the effects of methylation, chlorination, and deoxygenation of the tetracycline ring system on the ability of complex formation to slow the rate of alkaline degradation. Chlortetracycline is unusual in that repeated alkaline stability studies with complexes described with this antibiotic have failed to demonstrate greatly enhanced alkaline stability.

The resistance of certain of these derivatives to attack by alkali enables them to be prepared as stable solutions when the solution is sufficiently alkaline to avoid C·4 epimerization occurring in the pH range of 2–6 (2) and anhydro formation occurring under very acidic conditions.

Those derivatives showing enhanced alkaline stability experience little or no change in their susceptibilities toward acid degradation and toward C·4 epimerization in comparison to the uncomplexed antibiotic. The lack of significant change toward acid degradation is of particular interest because both alkaline degradation and acid degradation involve chemical alteration of the C·6 hydroxyl, together with other changes in ring C in the antibiotic. The fact that complexing can produce great changes in the alkaline reactivity of the C·6 hydroxyl and little change in the acid reactivity of the C·6 hydroxyl is an important clue to the mechanism of alkaline stability enhancement via complex formation. When 6-deoxy-6-demethyltetracycline is used as the antibiotic in this derivative, greatly enhanced stability toward acid (3) and alkali is observed.

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

February 1962 volume 51, number 2

Review Article

Colorants for Pharmaceuticals

By CHARLES J. SWARTZ and JACK COOPER

THE USE of colorants to improve the appearance or other characteristics of pharmaceuticals has been reported in early documents. In many of these, color was closely associated with diseases and their treatment. Since then, the artificial coloring of foods and medicines for various purposes has increased slowly until, in the middle of the 19th century, the discovery of the first synthetic dyestuffs provided the spark which brought into being a rainbow of new colors. Today, as a result of the imaginative research efforts of color manufacturers, a wide range of synthetic organic coloring agents has been made available for numerous industrial uses.

Though a minor consumer of colorants compared to the food or cosmetic industries, pharmaceutical manufacturers have a considerable interest in the use of dyes as colorants. Artificial colors are included in various dosage forms of pharmaceuticals for several reasons. Naturally, one such application is for the purpose of producing an eye-appealing product. Quite apart from esthetic enhancement and consumer appeal, it is often stated that certain colors elicit specific associated effects in some persons. In view of such psychological factors, color is often employed to create related taste responses in a patient prior to the ingestion of a medication. In order to increase the acceptability of various products, an organoleptically compatible color is selected with due consideration for the population group for which the drug is primarily intended.

Received from the Research Department, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Colorants have also been used in pharmaceuticals to conceal or modify undesirable visual impressions which are inherent to a particular preparation. In dosage forms containing active principles resulting from crude drug extraction techniques, differences in the color of the finished product occur as a result of the normal variation between crude drug lots. This point has been recently stressed by Tucker (1) who proposes the use of a color spray drying technique to minimize such differences. In certain tablet dosage forms, small variations in the method of manufacture or physical characteristics of ingredients will produce tablets with varying degrees of whiteness. Syrups and elixirs containing natural flavoring oils also exhibit batch variations in color. Therefore, the addition of artificial colorants provides the means by which a reproducible, uniform appearance of the finished product can be obtained.

Certain drug products contain added color to achieve a desirable cosmetic effect. Preparations for the treatment of skin conditions such as acne may, in addition to their therapeutic ingredients, also contain a skin-tone color to conceal the blemishes caused by the primary condition.

Knowledge gained in recent years has produced a tremendous increase in the use of drugs in veterinary medicine, both therapeutically and prophylactically. These drug products are often marketed by pharmaceutical manufacturers in the form of a "premix" which, prior to distribution to the ultimate consumers, will be mixed by the feed manufacturer with large quantities of animal or poultry feed. Some manufacturers

have colored these premixes artificially to give the feed manufacturer a visual aid in determining whether the medicated premix has been adequately dispersed throughout the finished feed.

One of the most important uses of dyes in drug specialties is for the purpose of identifying the drug in question. With the ever increasing number of drug preparations reaching the market it is readily understandable that any distinguishing feature which enables the physician, pharmacist, and nurse to identify a produce readily is essential medically and toxicologically. The importance of drug identification by color for these reasons has also been recognized by the Food and Drug Administration (2). The expense of special monograms and the limited variations in possible tablet shape also make the coloring of tablets an almost indispensable aid in the identification of drug products.

These several and varied uses of colors in medicinals emphasize the importance of dyes as used by pharmaceutical manufacturers. In the light of recent Food and Drug Administration rulings proposing to delist the great majority of dyes certified for drug use, it is quite clear that a thorough evaluation of the chemical and biological properties of such dyes is an urgent consideration.

CHEMISTRY OF COLORANTS

The current literature on the reactions of dyes in pharmaceutical dosage forms is rather limited as a result of the practice of discarding a dye if its characteristics in a particular system were not satisfactory. Formulators may soon find that they will no longer be able to replace dyes in dosage forms readily when they find that they are physically or chemically incompatible with some component of the composition. Instead, it will be necessary to determine the nature of the offending property and if possible, eliminate it.

Generally speaking, the colorants still available for use in pharmaceuticals can be divided into three major groups: synthetic dyes, lakes and pigments, and natural coloring principles. The basis for the distinction between dyes and pigments is a physical rather than a chemical property, with solubility usually employed as the criterion. Dyes are defined as colorants which are soluble in some commonly available liquid, such as water, alcohol, or oils (3). Those colorants which are essentially insoluble in such liquids or in any medium in which they are designed to be used are classified as pigments.

A specialized area of synthetic pigments of pharmaceutical interest is the class of lakes of the FD&C dyes. These insoluble materials are prepared from certified water-soluble dyes by combination with a basic aluminum or calcium radical and precipitation on a substrate of aluminum hydrate, thus forming an insoluble salt. In addition, there are numerous inorganic pigments such as titanium oxide, iron oxide, etc., which are completely insoluble in organic solvents and aqueous solutions. The constitution, properties, and biological significance of natural coloring principles have been the subject of an excellent review by Mayer (4), although such colors have been largely replaced by the certified synthetic colorants.

Synthetic Dyes.—As the list of certified synthetic colorants for pharmaceuticals threatens to become minimal, a consideration of the properties of those dyes remaining available should be undertaken. First, the properties of an ideal colorant for pharmaceuticals might be contemplated (5). These would include: (*a*) ability to withstand temperatures ranging from 10 to 110° or even higher, (*b*) must be stable within a pH range of 2–9, (*c*) must be stable to light over long intervals of exposure, (*d*) should be available in both an oil-soluble and water-soluble form, (*e*) should blend well with other colorants in various systems, (*f*) should resist oxidation and reduction, (*g*) color strength should be capable of standardization so that there is no batch to batch variation, and (*h*) the necessity of being without suspicion of carcinogenic activity.

Although there are currently no dyes that fulfill all of the above, many of these criteria can be met. Information concerning the chemical structures and physical properties of the certified dyes are available from numerous sources (5–13). In a paper prepared for the Perkin Centennial, Zuckerman (12) has discussed the structure and properties of present day certified colorants. From a chemical standpoint the certified FD&C colors fall into five classes: azo, triphenylmethane, indigoid, xanthene, and pyrazolone.

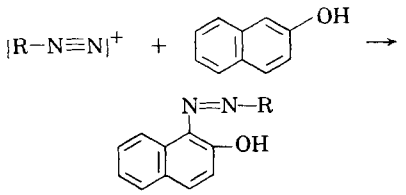
Azo dyes, however, comprise almost 90% of the total volume of certified colors used. A knowledge of their chemical structure can be employed to predict, to a fair degree, the physical properties of these colorants. The general formula for an azo dye is

$$R_1—N{=}N—R_2$$

R_1— is any aryl group

R_2— is a phenol, amine, or other group capable of coupling to a diazonium salt

The general coupling reaction involved in the formation of the finished dye would be (8)



Inskeep and Kretlow (14) have illustrated, in great detail, the general procedures for the preparation, purification, and blending of certified dyes of the azo type. The batch operation is carefully described in a flow sheet as well as through the use of structural formulas. It is pointed out that the presence of water solubilizing groups such as SO_3Na, COONa, and, to a lesser extent, the OH group, whether substituted on the diazonium component or the coupling component, increases the water solubility of the dye molecule. The absence of these groups gives molecular structures which exhibit true oil solubility. The manufacture of colorants of certified purity is a highly specialized branch of the dyestuff industry because extraordinary precautions and controls are necessary to eliminate objectionable side reaction products and metallic or other impurities. For example, the maximum amounts of lead and arsenic permissible are 10 and 1.4 p.p.m., respectively, in the FD&C colors and 20 and 2 p.p.m. for the D&C and Ext. D&C colors (15). The water, acids, alkalies, solvents, and intermediates used must all be the purest obtainable.

As noted by Zuckerman (12) the specific properties of a dye, such as fastness and chemical behavior, depend largely on the substituent groups. These groups are of two kinds: the chromophoric groups and the auxochromic or salt-forming groups such as the acidic or hydroxyl group. The chromophoric groups determine the chemical class to which the dye belongs and its oxidation or reduction potential. The auxochromic groups are largely responsible for the coloring properties and the action toward acids, alkalies, and light. An additional function of an auxochromic group is to stabilize the molecule so that the new molecular configuration has a greater resonance stabilization than the unsubstituted compound. This stabilization causes absorption in the longer wavelengths and results in a deepening of the color. Changes in dyes consisting either in complete or partial loss of color may occur for several reasons, which will be discussed under the heading of stability.

Since it is impossible to produce synthetic dyes commercially which consist of a single chemical entity, the importance of definitive analytical methods for the determination of these dyes is self-evident. The well established methods for the analysis of certified colors are given in "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists" (16). However, incomplete and side reactions during synthesis normally produce substances other than the principle component. Also, certain extraneous materials may be introduced during the process of isolation of the pure dye. Research conducted in the color laboratories of the U. S. Food and Drug Administration as well as in the laboratories of dye manufacturers has established for the majority of FD&C colors: (*a*) their chemical structure, (*b*) the identity of the isomeric and homologous colors, and (*c*) the identity of colorless organic constituents.

As a result, rapid and accurate methods for the quantitative analysis of all organic constituents expected to be present in a dye are now available. This then permits interested regulatory agencies to describe each color accurately and to determine whether each batch submitted for certification is equivalent to the reference standard material previously approved.

Jones, *et al.* (17, 18), were able to separate and determine the pure dye content of samples of triphenylmethane colors by a stepwise synthesis and analysis of all components of all products of the reactions. They were further able to differentiate each of the isomers of all of the various dyes by a variable reference technique (19).

Several investigators (20–30) have isolated and identified intermediates produced in the preparation of FD&C dyestuffs. In particular, the work of Link (20) presents a general procedure by which dyestuffs can be examined for the presence of uncombined intermediates. The procedure involves a chromatographic separation followed by a spectrophotometric identification of the uncombined intermediates. A technique for the purification of FD&C Red No. 2 suitable for use as an analytical reference standard was also described by Link (21). Methods suitable for the detection of six subsidiary colors in FD&C Red No. 2 are also presented.

Jones, Harrow, and Heine (28) undertook a thorough investigation of the composition of FD&C Blue No. 2. This color is prepared commercially by sulfonating indigo. Each of the possible symmetrical sulfonated compounds was prepared in addition to various disulfonated products and a chromatographic method of

separating many of the products was developed. A spectrophotometric method which was developed was found to be a much more precise method than titration with standard titanium trichloride for determination of pure color in FD&C Blue No. 2.

Of the FD&C colorants, the one distinct chemical class remaining is found in FD&C Red No. 3, an iodofluorescein derivative. A thorough study of a closely related series, the bromofluoresceins, was conducted by Graichen and Molitor (29) in 1959. It can be expected, however, that the chemistry of the iodofluoresceins is practically identical with that of the bromofluoresceins. A total of at least 10 halogenated products theoretically could be expected under the conditions employed for the iodination of fluorescein.

Thus, information is now available on the chemistry of the certifiable color additives and their subsidiary products formed through secondary reactions. This information should be expected to contribute materially to the clarification of legislation designed to evaluate certifiable color additives of the FD&C series.

Pigments and Lakes.—Approval by the Food and Drug Administration in 1959 of certified lake pigments for pharmaceutical uses has considerably enlarged the utility of this class of colorants. Previously, only titanium dioxide, purified carbon black, and carmine were approved by the FDA for food, drug, and cosmetic use. This development results in a significant step forward for the pigment field. The general manufacturing details of pigment preparations, together with their properties, have been reviewed by Anstead (31). He also includes a summary of the requirements of colors for cosmetics including aspects of relevant legislation in Great Britain and the United States.

An excellent review of the chemistry and physics of organic pigments is provided by Pratt (34). Of particular note is a special chapter in which emphasis is placed on a new interpretation of the significance of physical structure of pigments as related to color. This factor is of fundamental importance in the use of nondissolved materials. With dyestuffs, consideration of the relationship between color and structure has been largely restricted to the basis of chemical or molecular structure. In pigment technology, in addition to chemical properties, a study and measurement of particle size and shape, aggregation, molecular space relationships, and surface conditions are essential for total understanding and utility of these materials. As there are many applications for these materials in the food, drug, and cosmetic fields, Powers (9) encouragingly states that inorganic pigments have excellent light fastness and complete insolubility in solvents and aqueous solutions. He notes that the most important pigments are the iron oxides, chrome oxide greens, ultramarine blues and pinks, and the carbon blacks. A summary of the properties of the above is supplied. Mattiello (11) has compiled a book describing the chemical compositions, manufacturing processes, properties, uses, and methods of identification of pigments. The various chapters have been written by recognized authorities in the field of protective coatings and pigments, with each section being prefaced by an excellent brief theoretical discussion.

Zuckerman (32) briefly describes the use of lake colors in and on food products and notes that application will surely be found in many areas in addition to their use in foods. Lake colors are odorless and tasteless and are resistant to migration due to their insolubility. Jablonski (10) has reviewed the use of pigments and lakes in food products. He notes that until recently only lakes prepared from animal or vegetable coloring matters achieved widespread usage. With the exception of the use of titanium dioxide in tablet coating, reports on the use of pigments in lakes in pharmaceutical products are sparse. Tucker and co-workers (33), however, have reported on tablet color coating with pigments.

With few exceptions the natural organic coloring matters find little use in the pharmaceutical field. A comprehensive review of the significant chemical and physical properties of the natural colorants is supplied by Mayer (4). Various authors (6, 9) have commented briefly on their use in the drug field and summarized their application properties.

STABILITY OF COLORANTS IN PHARMACEUTICAL SYSTEMS

Although the use of synthetic dyes for imparting color to medicinals is obviously widespread in the pharmaceutical industry, investigations into the stability and stabilization of colors in these products have, until recently, received little attention by pharmaceutical researchers. With a few notable exceptions, the literature on the use of colorants for pharmaceuticals is so scattered and incomplete as to offer little assistance to a worker in the field of color stability as far as industrial application is concerned. The photosensitivity of these dyes, when incorporated into various formulations, represents a formidable

problem. Though this is a generally recognized difficulty, a conspicuous lack of information still exists in the pharmaceutical literature concerning the influence of light and heat on color fastness. This lack of data can be ascribed to several factors, among which are: (*a*) the unavailability of a suitable exaggerated light stability cabinet, (*b*) the unavailability of appropriate analytical techniques for measurement of color changes under the various circumstances, (*c*) the common practice of discontinuing the use of a specific color when found unsuitable in a given formulation without determining the nature of the objectionable characteristics, and (*d*) the treatment of such data as confidential within a particular company.

Aspects of Photostability.—Approaches to predicting the stability of colorants have been based on both chemical and physical measurements. Since the principle mode of reduction of color intensity results from photochemical reactions, interested individuals have attempted to utilize various exaggerated light testing techniques in an effort to predict the degree of photosensitivity of dyes contained in their products. However, although it is known that tests of this nature have become more prevalent, few reports exist in the pharmaceutical literature concerning their use. This may be attributed to the fact that the methods employed were not completely satisfactory or were highly specific (35). Experiments performed by Arny, *et al.* (36, 37), disclosed the effects of sunlight and diffused light on various pharmaceutical ingredients when stored in several types of glass containers. Of all the commercially available glasses studied, the authors found that amber glass afforded the greatest protection.

Although there is some lack of information concerning the photostability of pharmaceuticals, there appears to be an abundance of data available on the influence of light on textiles, dyes, and pigments (38–47). The necessity of having a standard light source is well recognized in these fields.

The most readily available source of radiant energy is sunlight, but Hadfield (47) has thoroughly delineated the unsuitability of sunlight or daylight as a source of light in testing color stability. He points out that the radiation received from the sun is subject to wide variation both in total intensity and in distribution of energy. Because of this, exposure tests to sunlight result in marked differences in relative effect for samples exposed to this light source. It has been reported by Appel and Smith (43) that by limiting the exposure of samples to between 9 a. m. and 3 p. m. on clear days, the low intensity radiation and high atmospheric humidity occurring at intervals during continuous exposure is avoided. This, they indicate, yields data that have been shown to be generally reproducible at different times of the year and in different parts of the United States. Despite the above efforts at standardization of sunlight exposure, the findings of other investigators appears to be at variance with the findings of Appel and Smith.

Attempts to measure the energy of the sun's rays and to make exposures for a definite number of energy units were inadequate in that the following factors were not taken in consideration: (*a*) variation of sun intensity from season to season and area to area, (*b*) daily cycles of change in temperature and humidity, (*c*) variations in spectral character of radiation during time of exposure, and (*d*) the presence of atmospheric impurities (48). In an endeavor to concentrate the sun's rays and thereby decrease the time required for fading exposure, lenses were utilized, though unsuccessfully, because of the excessive heat developed (49). In order to control, diminish, or eliminate the effect of these variables, various artificial light sources have been developed with a spectral energy distribution intended to simulate that of direct sunlight. Sources such as carbon arcs (50, 51), mercury vapor lamps (52), fadeometers (39, 41, 44), and tungsten filament lamps (42, 45) have been used to determine the light stability of colored textiles, dyes, and pharmaceuticals susceptible to photochemical reaction.

Since most pharmaceutical preparations are usually stored in retail pharmacies, storage areas of a warehouse, homes, hospitals, and doctors' offices, the amount of light in the ultraviolet spectrum of sunlight that penetrates through the glass store front or window panes is substantially decreased because of absorption by the glass. Husa (58) indicated that ordinary glass absorbed the shorter ultraviolet light as well as the longer infrared found in sunlight. This would suggest that the decomposition caused by sunlight passing through glass would be due to visible light and/or the ultraviolet and infrared rays which are nearest in wavelength to visible light.

In a report by Esselen and Barnby (59), light intensities outside and inside retail stores were measured and compared. The data obtained indicate that the outdoor readings were over 400 times the average reading taken on typical store shelves exposed to light of normal intensity.

Pharmaceutical products are very seldom exposed to direct sunlight and when they are, it is for a relatively short period of time. As a result, it was felt that a high intensity light source which would closely simulate ordinary room illumination would be more suitable for the study of dosage forms (35). This type of light source should approximate more closely the light falling on products kept in usual storage areas. Several studies to be discussed subsequently employed the above-mentioned light source as a standard for testing.

Cabinets for housing a suitable light source, whatever its nature, are available in a multiplicity of designs. Numerous patents (53–57) have been issued for various installations depending upon the desired end use. The basic design consists of a housing to contain the sample at a fixed distance from a light source. In some instances provisions are made for humidity and/or temperature control while, in other cases, only the character of the radiation is regulated. The degree of control varies with the intended end use of the data obtained but, in almost all situations, an attempt is made to extrapolate the information derived to some extended time interval.

Chemical and Physical Stability Considerations.—Certain general statements can be made about the chemical and physical behavior of certified colorants in pharmaceutical systems. Changes in dyes resulting either in complete or partial loss, or in alteration of color, may occur for several reasons. Some of the more common causes for the color change are exposure to light, the action of oxidizing or reducing agents, and the action of acids or alkalies. Peacock (7) has tabulated data on the resistance of the certified colorants to a wide range of reactive agents and conditions. The summary gives an indication of dye resistance to sunlight, to organic and inorganic acids, alkalies and some metallic salts, and to oxidizing and reducing conditions.

Inskeep and Kretlow (14) have also summarized the relative stabilities of the FD&C colorants. They note that the degree of fastness of various synthetic dyes may vary with the dye itself, the nature and intensity of light, the amount of moisture present, and the vehicle in which the dye is employed. They have presented the following general information:

"*A common property of dyestuffs is their ability to take up hydrogen with the formation of colorless compounds. In nitro and azo dyes, amino compounds are produced, whereas other classes are converted into so-called leuco-compounds which contain two more atoms of hydrogen than the original dye from which they are derived. They are reconverted into the original dye on oxidation.*

"*Most of the certified colorants are very unstable in the presence of reducing agents. The same is true in the presence of oxidizing agents, although the red colors are slightly more stable than the others. The most common type of reduction encountered in the use of FD&C colors involves the action of a metal such as tin or iron in an acid medium. The acid acts on the metal, liberating hydrogen, which in turn reacts with the dye, to bring about a reduction. The result is a loss of color.*

"*The certified colorants vary considerably in their action towards acids and alkalies. FD&C Blue No. 2, for example, fades rapidly in an acid medium; FD&C Red No. 3 is completely precipitated and should not be used in any product where acid is present. The azo dyes are comparatively stable in weak acids, but some of them should not be used in a basic environment.*"

Evenson (60) has studied the effect of light on FD&C Red No. 3 and D&C Red No. 21. His study has shown that the addition of small amounts of FD&C Blue No. 2 greatly accelerates the fading of FD&C Red. No. 3 in solutions. As a result, he points out that care should be taken to select mixture components of approximately equal resistance to light, if possible.

Several studies involving pharmaceutical systems have been reported by Lachman and colleagues (61–66). One such investigation was conducted in an effort to determine the light stability of 10 certified water-soluble dyes when used in tablet dosage forms (62). No effort was made in the study to elucidate the specific fading mechanisms of the several colors; rather, an attempt was made to determine the relative stability of the colors under normal and exaggerated illumination. The relationship between fading and dye structures as well as fading and light intensity was discussed. The extent of dye fading beneath the surface of the tablet was also measured. The authors concluded that the fading rate is most rapid during the initial stages of irradiation. Of significance is the observation that the exaggeration of color fading produced by the intensified illumination source employed did not follow a particular pattern for the dyes studied.

In another study using identical exaggerated lighting conditions, Lachman (63) evaluated the relative light fastness of several water-soluble dyes and their corresponding lakes. Employing initial fading rate constants as criteria for de-

termining light fastness, the lakes studied exhibited less photostability than the dyes in the tablet systems studied. As an example of the variations in data that may result when different lighting systems are employed for exaggerated testing, Tucker (33) reported that enhanced light stability resulted from the use of lakes as colorants for pan coated tablets. There are no established standardized conditions for light stability testing in the pharmaceutical industry, and correlation of data is hardly possible. Thus, literature reports such as the aforementioned pair must necessarily be treated individually. It must also be remembered that the lakes were applied, in one instance, in conjunction with pan coating techniques and, in the other case, the lakes were blended directly into a granulation prior to compressing tablets.

In another study the influence of temperature and pH on the surface color and total dye content of tablets colored with certified dyes was investigated (66). Samples of tablets colored with three dyes and buffered at several pH levels were stored at elevated temperatures and tested for both residual dye content and surface fading. The results of the study disclosed no relationship between the fading at the surface of the tablets and the actual loss in colorant concentration at elevated temperatures.

The same authors have also evaluated the effect of ultraviolet absorbers on the photostability of colored tablets (65). These studies were performed under light sources simulating the spectral energy distribution of sunlight and normal and exaggerated room illumination. The use of the ultraviolet absorber was most effective against color fading in tablets exposed to simulated sunlight. Signore and Woodward (67) studied the stabilizing effects of ultraviolet absorbers such as 2-hydroxy-benzophenone derivatives on dyes in solution. They found that significant protection was afforded to selected dyes in 50% ethanol solutions irradiated in the presence of low concentrations of U.V. absorbing materials. However, U.V. absorbers do not universally protect all dyestuffs in solution as has been illustrated in papers by Hardy and Coleman (68) and Gantz and Sumner (69).

The protective influence of glass of various spectral transmission characteristics on the light-induced fading of colored tablets has been investigated by Swartz (64). The apparent rates of fading of the tablets irradiated through the various glass samples were determined following exposure of the tablets to intensified fluorescent illumination.

The observation that common pharmaceutical materials may react with dyes to promote fading in solution prompted Scott, *et al.* (70), to investigate the color loss of certified dyes in the presence of nonionic surfactants. The retention of color in aqueous buffered solutions containing certified dyes and nonionic surfactants was measured after 14 days storage at elevated temperatures. In all but four of the 30 systems examined, accelerated fading was noted over that obtained with solutions of the dye alone. The intensity of this effect was found to vary from one dye-surfactant system to another and was not generally predictable. Kuramoto and co-workers (71) determined the stability of FD&C Blue No. 2 in a buffered aqueous solution in the presence of several commonly employed pharmaceutical materials. They found that sugars such as dextrose, lactose, and sucrose increase the rate of fading, whereas sugar alcohols such as mannitol and sorbitol do not appreciably affect the rate. Numerous antioxidants were also tested in the system and found not to appreciably retard the fading. By means of phase studies using cloudpoint titration techniques, Lachman (72) studied the interaction between several quaternary ammonium compounds and certified dyes. It was demonstrated that three commonly employed cationic surfactants formed insoluble complexes with FD&C Red No. 1, FD&C Blue No.1, and D&C Yellow No. 10. Although the report was limited to data on only three certified dyes, the same phenomenon was found to occur with several other dyes.

Another study concerning itself with the mechanism of color fading in pharmaceutical formulations was presented by Garrett and Carper (73). This paper reported on the color stability of a liquid multi-sulfa preparation by kinetic investigation of the thermally accelerated degradation of the color. The colorant employed was a combination of FD&C Yellow No. 6 and D&C Red No. 33. The authors did not attempt to determine the actual mechanism of the reaction of the ingredients which were responsible for fading. They were principally interested in predicting the extended shelf color stability of a formulation in a brief period.

Though of less immediate value to workers in the pharmaceutical sciences, numerous reports have appeared in the literature of the textile and paper processing industries concerning dye stability. A group of the more pertinent of these will be commented upon in the following paragraphs.

A study of the oxidation of the azo dyes and

its relationship to light fading was carried out by Desai and Giles (74). They employed various oxidizing reagents including ceric sulfate, potassium dichromate, and hydrogen peroxide to disrupt the azo group in an effort to find if any correlation exists between resistance to oxidation and light fastness on textiles. However, following extensive experimental trials, the authors concluded that it seemed unlikely that a chemical agent could be found to fade dyes in the same manner as light exposure and thus be suitable for a rapid and reproducible screening test.

Atherton and Seltzer (75) attempted to correlate the fastness of dyes in cellulose acetate films to dye properties when deposited on textiles. They based their experiments on measurements made over the initial stages of the reaction in order to minimize the possibility of reactions between the dye and its decomposition products. The light source employed was an enclosed carbon arc projector. A series of simple monoazo dyes were selected because of their ease of synthesis, low light fastness, and colorless decomposition products. The results of these experiments did not permit the correlation hoped for. However, the workers were able to determine the influence of the chemical nature of substituent groups and their position relative to the azo linkage on the light fastness of the parent compound. This permitted them to propose a relationship between chemical structure and order of light fastness of these dyes under the specific conditions studied.

Other authors attempted to determine the influence of the substrate on the light fading of azo dyes and the influence of the physical state of the dyes upon their fastness properties in dyed textiles (76–79). Following a review of the data in every instance, only very limited correlations were possible. Desai and Vaidya (80) studied the action of light on certain triphenylmethane dyes, and by the use of both chemical and spetrophotometric methods were able to determine the photodecomposition products. They further showed that the same photochemical changes take place when the dyes are exposed to the action of different light sources. Various aspects of the fading of colored materials as a result of temperature, humidity, and light have been reviewed by Cunliffe (81), Morton (82), Taylor and Pracejus (83), and Barker (84). A review of the mechanisms of the free radical reactions which might take place in photosensitized oxidations of colorants in solutions has been reported by Weiss (85).

Rather than base the effects of light on dyes in solution on a systematic accumulation of practical data as has been done above, Bowen (86) has attempted to apply theoretical concepts in photochemistry to help clarify mechanisms in fading reactions. He has noted that many of the photo reactions of dyes have been interpreted as "electron transfer" reactions and suggests that experiments to elucidate some of the following properties would be helpful: "(*a*) precise measurements of the effects of oxygen (even in traces) on the photochemical change; (*b*) similar measurements with deliberately added oxidizing and reducing agents; (*c*) determinations of the temperature coefficient of photochemical reactions, without and with air present, to throw light on the nature of the processes limiting the efficiency; (*d*) examination of the effect of moisture on the reactions, in case liquid diffusion of substances should control the rate; (*e*) precise work on the detailed shapes of absorption bands of dyes both in solution and solid systems which might lead to a fuller understanding of the nature of the light absorbing process."

A more complete knowledge of the above should permit the active application of theory to the photodegradative processes of dyes.

APPLICATION PROPERTIES OF COLORANTS

It has been pointed out earlier in this report that quantitative data on the light and thermal stability of the certified dye is not readily available in published reports. Nevertheless, some information can be obtained from general reviews prepared by the various dye manufacturers (3, 6, 7) but these data are only qualitative in nature. Scott (70) has observed that the interactions between dyes and other pharmaceutical reagents leading to poor color stability in finished products have received little attention.

An excellent bibliography on color matching and its practical aspects has been prepared by Peacock (87). Color matching in the cosmetic industry is discussed by Stearns (88) who concludes that "color matching is very definitely an art, and the man who does color matching of cosmetics must be a highly skilled artisan." To give credence to this he states that there are 32 important factors involved in the judging of color effects, all interacting to various degrees.

The solubility properties of certified dyes are discussed in numerous trade articles. Upon observation of these values, variations in results that cannot be attributed exclusively to experimental error are sometimes found. Zuckerman

(89) determined that the maximum solubility of the dyes, at constant temperature, varied with with the quantity of excess solute and proceeded to develop a procedure for determining the maximum solubility of all the water-soluble FD&C colorants based on this.

An excellent review entitled, "Colour and Its Measurement" (90) has summarized many newer instrumental techniques of color measurement as applied to the textile industry. The bibliography contains 45 references in the general field of color perception, color application, and measurement.

THE REGULATION OF COLOR ADDITIVES

Following the discovery of mauve in 1856 by Sir William Perkin, the development of available colors with varied hues increased rapidly. For many years little attention was paid to the toxicology of these substances but by 1900 Congress was ready to appropriate funds to enable the Secretary of Agriculture to investigate the nature of "coloring matters" in order to establish the principles enabling their safe use in foods, drugs, and cosmetics. Before the passage of the Federal Food and Drug Act of 1906, seven colors were accepted as harmless and a voluntary system of certification based upon physiologic tests was set up. During the 32 years which elapsed before the new Food, Drug, and Cosmetic Act replaced the 1906 law, an additional 15 colors were certified for food, drug, and cosmetic use.

The 1938 Act provided more extensive and elaborate provisions for regulating the use of color additives with tests for toxicity, purity, stability, and local irritation. It also required that the structure of certified colors must be known and the purity determined by appropriate analytical methods. Certification was now divided into three classes: FD&C colors for foods, drugs, and cosmetics; D&C colors for drugs and cosmetics, and Ext. D&C colors for external use only.

Under the powers granted the FDA, decertification of previously approved colors has become increasingly frequent in recent years as more intensive pharmacological testing was applied to these substances. In addition, attitudes resulting from the controversy involving the safety of food additives were reflected in an increasingly critical and cautious attitude toward the use of color additives. In a report discussing Bill S2197 (Color Additive Amendments of 1959) Senator Hill (91) urged the replacement of the previous color additive regulations with the new "sound and uniform system for the listing of color additives of any kind which may be safely used in foods, drugs, or cosmetics subject, when necessary, to appropriate tolerance limitations and other conditions of use. . . ." Senator Hill was emphasizing the importance of the tolerance provision as an answer to a Supreme Court decision which defined the "harmless" principle (of the 1952 Act) as meaning harmless regardless of the quantity of the coal-tar color which is being used.

Williams (92), in analyzing from a legal point of view the color additive bills submitted to Congress, showed concern for the hazards associated with the procedures for listing and exempting safe color from certification. The absence of a practical jurisdictional test which would exclude from the statute colors known to be harmless contributed a burdensome character to the procedural requirements of the proposed legislation. Schramm (93), in a similar review of the bills before Congress in 1959, stated that the basic principles involved in these bills met the approval of the Certified Color Industry Committee. An objection of the Committee to the mandatory requirements for analytical methods as a condition for listing a color additive was resolved in the Senate bill on the basis of an industry and Department of Health, Education, and Welfare discussion. The Committee also objected to the specific inclusion of cancer since there was no logical reason to separate this pathology from other adverse physiological effects. Dohl (94), in discussing the proposed bills from the point of view of the pharmaceutical industry, while objecting to the Delaney Cancer Clause and to several less important aspects of the legislation, nevertheless supported the Senate bill as better than no legislation at all. Similar support, including the reminder that the pharmaceutical industry is accustomed to control, came from Goodrich (95).

On July 12, 1960, the Color Additive Amendments of 1960 became law and, following the expiration of a transitional period, all colors will become subject to uniform statutory rules. The disappearance of the term "coal tar colors" will not be mourned while its more inclusive replacement "color additives" must still find its place in the everyday vocabulary of pharmacy and the pharmaceutical industry. A section by section analysis of the new law has been published and should be very helpful to those responsible for conformance with the provisions of the law (96). Briefer commentaries (97, 98) reviewing the overall significance of the law have also made their appearance in literature.

Failure of the pharmaceutical industry to demonstrate the safety of D&C colors by 1963 will result in automatic decertification of these colors unless an extension is granted on the basis of studies initiated but not completed before the deadline. Many product development pharmacists and control chemists are of the opinion that permissible FD&C colors are inadequate for all pharmaceutical requirements. It is necessary to recognize the fact, and many pharmaceutical executives have not done so, that eliminating the use of colors in tablets, syrups, elixirs, etc., will not necessarily provide uniform, colorless, products. Variations in raw materials and intermediates are difficult or costly to control and lack of product uniformity will undoubtedly engender complaints. Significant protection against change in product appearance due to aging could also be provided by suitable coloring agents. The new color additives law regulates the production of pharmaceuticals in an important way and industry may find it advisable to invest in the acquisition of scientific information in order to assure compliance.

A remarkably thorough compilation and review of legislation regarding the available colorants for use in most of the nations of the world has been assembled by J. PlaDelfina (99, 100). In addition, this comprehensive report discusses the chemical structure and reactivity, toxicology, and pharmaceutical applications of all colorants available for both internal and external use. The review is appended by a series of outlines in the form of a catalog which summarizes and also completes the text making it useful for those not very familiar with the field. The six-column summary lists the chemical classification and structure, commercial and legal aspects, as well as pharmacologic data on all the material cited in the paper.

CONCLUSION

Research pharmacists and others concerned with the formulation of pharmaceutical products and the avoidance of confusion and errors in manufacturing and dispensing are dismayed and alarmed at the nonchalance with which many scientists and executives in the pharmaceutical industry have accepted recent decisions involving the decertification of important colorants. Any degree of reflection on the realities of a situation permitting only "colorless" or "naturally colored" pharmaceuticals, as has been blithely proposed by some, would bring into focus a host of new problems of disturbing significance. It is impossible to conceive of responsible individuals experienced in the development of pharmaceutical products who would solve problems at the expense of present or potential risk to patients. Scientists should, however, be prepared to fight off ill-conceived decisions based upon unknown fears or political advantages.

In spite of the lengthy history of the use of colorants in pharmaceuticals, a review of the literature brings out the fact that relatively little attention has been given to investigations of the behavior of colorants in pharmaceutical systems. Basic studies are needed in the field of chemical reactivity of colorants with other components of medications, the stability of individual or mixed colorants in varying environments, and the relative toxicity of color additives. As with all substances entering into biological systems, it is necessary to relate the quantity of agent administered with the response. Toxicology, like pharmacology, is dependent upon posology.

As is evident from this review, the research pharmacist planning investigations of the physical and chemical properties of colorants can lean upon the experiences of scientists in other fields whose experiments have yielded knowledge applicable to the use of such substances in drugs. The basic chemistry of dyestuffs, the application properties of colorants, and instrumentation for measuring color and color changes have been probed with concentrated intensity by workers in food processing, textiles, photography, and protective coating. The fruit of their labors provides a solid foundation for the development of needed knowledge in the formulation of pharmaceuticals.

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Solid State Stability of Some Crystalline Vitamin A Compounds

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Theoretical and experimental studies relating the relative stability of solid vitamin A derivatives to their melting points have shown that, in general, resistance toward degradation increases with fusion temperature. This is attributed to the part that crystal lattice energy plays in stabilizing these compounds, and is supported by the fact that the solution stabilities of the derivatives appear to be generally comparable. Of the several compounds studied, vitamin A benzhydrazone and vitamin A succinate triphenylguanidine salt exhibited the greatest stability.

THE PRESENT investigation has been concerned with the experimental examination of the relationship between fusion temperature and the rate of chemical degradation of certain susceptible solid drugs. Although it has been known that the stability of many solids is closely related to their melting points, rational application of this condition to stabilization of drugs has not been generally recognized by pharmaceutical investigators, and therefore has been quite limited. Specifically, several vitamin A derivatives of widely differing melting points have been prepared and their relative stability evaluated. The compounds studied in detail included vitamin A acetate, vitamin A benzhydrazone, vitamin A nicotinate, vitamin A phthalimido-N-acetate, vitamin A succinate triphenylguanidine salt, and vitamin A 3,4,5-trimethoxybenzoate.

The stability of organic compounds to degradation in the solid state is intimately related to the strength of the crystal lattice. Since the forces between molecules in a crystal are small compared with the energy required to break bonds, liquefaction normally occurs before degradation begins. As a general rule, reactions proceed at a much faster rate in liquids and in solution than in solids; consequently the melting point of a compound is a very important factor in determining stability.

The surfaces of higher melting crystals are a strong barrier to penetration of external agents responsible for degradation of chemicals in the dry state. The resistance of a crystalline compound to fusion can therefore be taken as a crude, but useful, index of its general stability.

Received April 20, 1961, from the School of Pharmacy, University of Wisconsin, Madison 6.
Accepted for publication June 26, 1961.
This study was supported in part by a grant from the Roche Laboratories, Nutley, N. J.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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Past Work on Degradation of Solids.—Most studies on reactions which occur in the solid state have dealt with inorganic solids and, in particular, with oxidation taking place at the surface of metals. Nearly all organic compounds, including drugs, are apparently essentially stable within their crystalline structure. Degradation, when it is observed, usually occurs in the surface film phase. Bawn (1) points out that decomposition of solid explosives, for example, is usually accompanied by the formation of a liquid phase at temperatures below the normal melting point of the pure solid. As a consequence, there is a rapid increase in the reaction rate. In the absence of melting, the formation of product molecules is assumed to induce a strain in the crystal which produces cracks and thus forms fresh surfaces on which decomposition can occur. Degradation of explosives usually leads to the formation of gaseous products. The rate of the process can therefore be most conveniently followed by measuring the pressure developed in a constant volume system. A sigmoid shaped pressure-time curve is often obtained experimentally.

Wobbe and Noyes (2) studied the solid state decomposition of anhydrous oxalic acid into gaseous formic acid and carbon dioxide at temperatures just below its melting point. The reaction was again shown to occur largely on the surface of the crystals. When small amounts of solid were used, the reaction could be better represented by a zero-order than by a first-order equation. For large amounts of solid, the constants were always high, however.

Garrett (3), studying the solid state decomposition of aspirin anhydride, found that the degree of degradation could be qualitatively correlated with the amount of liquefaction in the samples at the various temperatures studied.

Micronization, drying, and avoidance of contact with alkali increased the thermal instability of the anhydride. The unusual observation that small crystals were more stable than larger ones was explained by the fact that the large crystals were probably solvates or contained trapped solvents.

Past Work on the Stability of Vitamin A.—Details of the early work on the stability of vitamin A, particularly in the form of oily concentrates, have been described elsewhere (4–6) and will not be discussed extensively here. Certain aspects of this work are, however, pertinent to the present investigation.

For many years after its isolation from fish liver sources no crystalline derivatives of vitamin A were available. It was not until 1935 that Hamano (7) succeeded in crystallizing the first derivative of the vitamin, vitamin A β-naphthoate. Vitamin A alcohol itself was not obtained in the form of solvent-free crystals until 1942. In 1942, Baxter and Robeson (8) prepared crystalline vitamin A acetate, vitamin A palmitate, and divitamin A succinate; and they studied the relative stability of these compounds in the solid state. Thin layers of the crystals were exposed to air at 5° in darkness. The percentage decomposition at various times was determined by the percentage decrease in the extinction coefficient. Vitamin A β-naphthoate was included in the stability study. The apparent order of stability of the compounds studied was: vitamin A acetate, vitamin A β-naphthoate, vitamin A palmitate, vitamin A succinate. The greater stability of vitamin A acetate was attributed to its crystal size. Apparently other factors were involved, however, for the authors could not explain with this hypothesis the exceptional stability of vitamin A β-naphthoate.

In recent years studies on the solid state stability of vitamin A and its compounds have been largely concerned with the effectiveness of various antioxidants. Some work has also been done on the preparation of protein complexes (9, 10), inclusion compounds (11, 12), and coatings (13–15). Few new crystalline derivatives of vitamin A have been reported.

The mechanism by which crystalline vitamin A compounds degrade has not yet been completely elucidated. This degradation is probably similar to the changes which occur in the drying of oils and in the polymerization of similar conjugated polyolefinic molecules. Two related types of reactions probably are involved: (*a*) free radical catalyzed autoxidative polymerization occurring at the surface of the crystal, and to as great a depth in the liquid film covering the crystal as oxygen can penetrate, and, less likely, (*b*) polymerization involving carbon-carbon linkages beyond the depth of penetration of oxygen.

Reinstein (16) has studied the degradation of vitamin A alcohol, vitamin A acetate, and vitamin A palmitate at 25° and 90° in heptane and ethanol, in the presence and in the absence of air. In the presence of air, the two esters degraded at similar rates, while the alcohol form decomposed approximately three times faster than the esters. The order of the reaction with respect to vitamin A was 1.3 to 1.5, indicating that very concentrated solutions lose vitamin A at a much faster rate than dilute solutions. When air was excluded, the rate of degradation was barely detectable, indicating that the degradation of vitamin A in heptane was essentially oxygen initiated.

EXPERIMENTAL PROCEDURE

Reagents.—Crystallize vitamin A alcohol was obtained by base-catalyzed hydrolysis of vitamin A acetate (Hoffmann-LaRoche). Benzhydrazide, potassium phthalimide, 1,2,3-triphenylguanidine, and dichloromethane were used as obtained (Eastman Organic Chemicals Co.). Chloroacetyl chloride and methyl formate (Eastman Organic Chemicals) were redistilled. Skellysolve A, Skellysolve B, and petroleum ether were used without purification. Manganese dioxide was obtained from General Metallic Oxides Co. All other solvents and chemicals were reagent grade.

Apparatus.—All ultraviolet spectra were determined in chloroform on a Cary recording spectrophotometer model 11MS. Infrared spectra were determined using a Beckman model IR-5 double-beam infrared spectrophotometer. Melting points were determined in capillary tubes, using a 3-inch immersion thermometer.

Analyses.—The microanalyses were performed by Dr. Stephen M. Nagy, Microchemical Laboratory, Massachusetts Institute of Technology.

Synthesis of Compounds

Vitamin A Benzhydrazone.—This compound precipitated when a solution of benzhydrazide in methanol was added to vitamin A aldehyde. The vitamin A aldehyde was prepared from vitamin A alcohol by oxidation with manganese dioxide, a method first used by Ball, *et al.* (17).

Activated manganese dioxide (28 Gm.) was added to a solution of 4 Gm. vitamin A alcohol in 40 ml. petroleum ether. The solution was agitated from time to time for 20 hours at room temperature. The ether solution was filtered off and evaporated to a yellow oil. Benzhydrazide (1.9 Gm.) dissolved in 50 ml. methanol was added to the vitamin A aldehyde. After a few minutes crystals formed in the solution. The crystals were filtered off, washed with petroleum ether, and recrystallized from methanol, m. p. 181–182°.

Anal.—Calcd. for $C_{27}H_{34}N_2O$: C, 80.5; H, 8.5; N, 7.0. Found: C, 78.9; H, 8.7; N, 7.1.

Vitamin A Nicotinate.—This ester was prepared by alcoholysis from Vitamin A alcohol and methyl nicotinate in Skellysolve B, with sodium methoxide as catalyst.

Crystalline vitamin A alcohol (18 Gm.) and crystalline methyl nicotinate (10 Gm.) were dissolved in 200 ml. Skellysolve B. Sodium methoxide (0.1 Gm.) was added and the mixture was heated at 60° for 2 hours. Skellysolve B was collected as it distilled in a Barrett trap, and fresh solvent was added to the reaction vessel as required. The flask was protected from light and moisture, and nitrogen was bubbled into the solution throughout the reaction. After the heating period the solution was extracted several times with water until the washings were neutral to litmus. The Skellysolve layer was dried over sodium sulfate, filtered, and refrigerated at 0°. Crystals formed in the solution overnight. The yield of vitamin A nicotinate was 8.5 Gm. An additional 4 Gm. of nicotinate was obtained from the mother liquor after concentration. Vitamin A nicotinate was recrystallized from methyl formate, m.p. 93–94°.

Anal.—Calcd. for $C_{26}H_{33}NO_2$: C, 79.7; H, 8.5; N, 3.6. Found: C, 77.1; H, 8.2; N, 3.8.

Vitamin A Phthalimido-N-acetate.—Vitamin A chloroacetate and potassium phthalimide afforded this compound in dimethylformamide.

Vitamin A chloroacetate was prepared by a modification of the procedure of Baxter and Robeson (8) for preparing esters of vitamin A from acid chlorides. Chloroacetyl chloride (4 Gm.) dissolved in 50 ml. dichloromethane was added dropwise from a dropping funnel into a solution of vitamin A alcohol (10 Gm.) in 100 ml. dichloromethane and 30 ml. pyridine. Nitrogen was bubbled into the solution throughout the reaction, and the mixture was kept at 0–5°. After the acid chloride was added, the solution was stored in the dark at room temperature for 2 hours. A saturated aqueous solution of oxalic acid was added and the solution was extracted with petroleum ether. After several washings with oxalic acid solution the ether layer was washed with water until neutral to litmus, then dried over sodium sulfate. The solvent was removed under reduced pressure and nitrogen, and potassium phthalimide (3 Gm.) dissolved in 50 ml. dimethylformamide was added. The solution was stored in the dark at room temperature for 12 hours. Water (100 ml.) was added, and the solution was extracted with ethyl ether. The ether layer was washed several times with water, then with 5% sodium hydroxide solution, and finally with water again, until neutral to litmus. The ether solution was dried over sodium sulfate, filtered, and refrigerated at 0°. Vitamin A phthalimido-N-acetate crystallized from the ether solution was filtered off and recrystallized from anhydrous ethyl ether, m.p. 111–112°.

Anal.—Calcd. for $C_{30}H_{35}NO_4$: C, 76.1; H, 7.5; N, 3.0. Found: C, 75.8; H, 7.6; N, 3.4.

Vitamin A Succinate Triphenylguanidine Salt.—Vitamin A succinate half ester was prepared by heating vitamin A alcohol and succinic anhydride in pyridine. 1,2,3-Triphenylguanidine was added to the ester in saturated ether solution and the salt precipitated.

Vitamin A alcohol (11.5 Gm.) and succinic anhydride (4.0 Gm.) were dissolved in 50 ml. pyridine in a flask protected from light. The solution was heated at 75° for 1 hour. Nitrogen was bubbled through the solution throughout the reaction while the mixture was cooled to room temperature. The pyridine solution was transferred to a low-actinic separator and 50 ml. petroleum ether was added. The ether solution was extracted four times with 75-ml. portions of saturated aqueous oxalic acid solution, then washed with distilled water until washings were neutral to litmus.

Five per cent sodium hydroxide (40 ml.) was added in 10-ml. portions with shaking. The ether layer contained unreacted vitamin A alcohol and was discarded, while the aqueous layer contained the sodium salt of vitamin A succinate. This was washed four times with 50-ml. portions of petroleum ether, which were discarded. The aqueous layer was acidified with saturated oxalic acid solution, and the vitamin A succinate was taken up with 50 ml. petroleum ether. The ether layer was washed again with 50-ml. portions of distilled water until neutral. It was then dried over anhydrous sodium sulfate and filtered. The ether solution was evaporated to a yellow oil by distillation from a 40° water bath under reduced pressure and nitrogen. The product obtained (13 Gm.) had an ester peak in the infrared at 5.81 μ. There was no alcohol peak.

Triphenylguanidine (4 Gm.) dissolved in 125 ml. anhydrous ether was added to 5 Gm. vitamin A succinate dissolved in 25 ml. ethyl ether. A white precipitate formed within 2 minutes at room temperature. After 15 minutes the precipitate was filtered off, washed several times with anhydrous ether, then dried in a vacuum desiccator, m.p. 140–140.5°. Vitamin A succinate triphenylguanidine salt was recrystallized from methyl formate without change in melting point.

Anal.—Calcd. for $C_{43}H_{51}N_3O_4$: C, 76.6; H, 7.6; N, 6.2. Found: C, 75.9; H, 8.0; N, 6.2.

Vitamin A 3,4,5-Trimethoxybenzoate.—This ester was prepared in the same way as vitamin A nicotinate, i. e., by alcoholysis from vitamin A alcohol and methyl-3,4,5-trimethoxybenzoate in Skellysolve B, with sodium methoxide as catalyst.

When crystals of vitamin A 3,4,5-trimethoxybenzoate were obtained from the Skellysolve B solution from which they were crystallized, they were washed with Skellysolve A, then recrystallized from Skellysolve B, m.p. 85–86°.

Anal.—Calcd. for $C_{30}H_{40}O_5$: C, 75.0; H, 8.4. Found: C, 75.0; H, 8.2.

Stability Studies

Solid State Studies.—Samples of 3×10^{-5} moles of each crystalline vitamin A compound were weighed into 1-dr. vials. The vials, open to air, were placed in a thermostated oven at 50 ± 1°. At intervals, sample vials were withdrawn and their contents transferred quantitatively into 100-ml. volumetric flasks. Samples of vitamin A nicotinate, phthalimido-N-acetate, succinate triphenylguanidine salt, 3,4,5-trimethoxybenzoate, and acetate were scanned in the ultraviolet region from 400 to approximately 280 mμ. All these compounds exhibited a peak at 333 mμ in chloroform, the only readily available solvent in which all the derivatives

were soluble. Vitamin A benzhydrazone solutions were scanned in the visible region, from 500 to 350 mμ. The vitamin A benzhydrazone peak was at 398 mμ. As degradation proceeded, the decrease in height of the respective peaks was noted.

Solution Studies.—Samples of each vitamin A compound were dissolved in chloroform, and aliquots containing 4×10^{-4} moles/L. were pipetted into 100-ml. volumetric flasks. The flasks were stored open to air in a dark cabinet. Periodically samples were removed, the proper dilution made, and the samples analyzed in the same way as in the case of the solid state studies.

RESULTS AND DISCUSSION

For the purpose of the present investigation the synthesis of a large number of derivatives was attempted. These derivatives are listed in Table I. Because of the extreme susceptibility of vitamin A to isomerization, polymerization, dehydration, and other chemical changes, these attempts led, in many instances, to reaction products contaminated with side products. Many of the derivatives successfully prepared were hygroscopic and, therefore, unsuitable for the planned studies. Stability investigations were limited to those compounds, marked with a superscript *a* in Table I, which were recoverable as sharply melting, nonhygroscopic crystals.

Relative Stability of the Derivatives in the Solid State

Results of studies on the relative stabilities of several vitamin A derivatives in the solid state are shown in Fig. 1. The data collected were based on spectrophotometric determinations of the absorption peak in the ultraviolet of separate solid samples kept for varying periods of time at 50°. Since there was a loss of less than 33% of the vitamin during the period of study, it is presumed that the data provide a fair representation of the stabilities of these derivatives.

No serious attempt was made to control the size of the crystals employed in these studies. The compounds, listed in order of decreasing crystal size were: vitamin A nicotinate, vitamin A acetate, vitamin A 3,4,5-trimethoxybenzoate, vitamin A phthalimido-N-acetate, vitamin A benzhydrazone, and vitamin A succinate triphenylguanidine salt. No marked correlation is apparent between crystal size and stability for these compounds, as shown in Fig. 1.

In the discussion of possible mechanisms of degradation of vitamin A it was postulated that degradation probably occurs almost exclusively in a liquid film at the surface of the crystal. A difference in reactivity of substances in the solid and liquid state is to be expected since there is a difference in internal energy between the two states equal to the latent heat of fusion (1). The relative amount of material in the liquid state may be expected in these instances to determine, in some large measure, the rate of degradation. The fraction of material in the liquid state is related to the melting point of the pure crystalline solid by the following equation

$$\ln x_l = \frac{-L_f}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)$$

Table I.—Synthesized Derivatives

Vitamin A Compound	Melting Point, °C. Crystalline	Melting Point, °C. Amorphous
Divitamin A fumarate[b]	...	...
Divitamin A diglycol carbonate[b]	...	...
Divitamin A pyromellitate[b]	...	...
Vitamin A benzhydrazone[a]	181–182	...
Vitamin A *p*-phenylenediamine, Schiff's base	155–156	...
Vitamin A succinate half ester[c]	...	...
Calcium salt	...	225
Magnesium salt	...	225
Procaine salt[d]	...	...
Ethylenediamine salt[d]	...	98–99
Triphenylguanidine salt[a]	140–140.5	...
1,4-Butanediamine salt[b]	...	...
Guanidine salt[b]	...	...
1,2-Propanediamine salt[b]	...	...
Thiamine salt[b]	...	...
Gramine salt[d]	...	130–132
N,N′-Dibenzylethylenediamine salt[b]	...	...
Tris-(hydroxymethyl)-aminomethane salt[b]	...	...
p-Xylyldiamine salt[d]	...	...
Arginine salt[d]	...	...
Glycine ethyl ester salt[d]	...	...
Glycine amide salt[b]	...	...
Vitamin A phthalate half ester[c]	...	...
Calcium salt	...	225
Ethylenediamine salt[d]	...	150
Hydrazine salt[d]	...	134
Triphenylguanidine salt[b]	...	...
Procaine salt[d]	...	82
1,2-Propanediamine salt[b]	...	...
Thiamine salt[b]	...	...
p-Xylyldiamine salt	...	122
N,N′-Dibenzylamine salt	...	...
Glycine amide salt[d]	...	117
Vitamin A chloroacetate	...	...
Vitamin A phthalimido-N-acetate[a]	111–112	...
Vitamin A dimethylglycinate	...	...
Tartrate salt	...	70–71
Gentisic acid salt	...	66–67
Maleic acid salt	...	94–95
Gallic acid salt	...	...
Vitamin A oxamido urethane	...	114
Vitamin A nicotinate[a]	93–94	...
Vitamin A isonicotinate[c]	...	...
Vitamin A 3,4,5-trimethoxybenzoate[a]	85–86	
Vitamin A *p*-aminobenzoate[c]	...	...

[a] Compounds used in this study. [b] Not obtained. [c] Obtained as an oil. [d] Hygroscopic.

where x_l is the mole fraction of the solvent, here the liquid phase, L_f is the molar heat of fusion, R is the gas constant, and T_m is the melting point of the pure solid.

It would appear for these systems then, that the relative rates of degradation of series of similar solid derivatives measured at any temperature, T, would be such that the logarithm of the rates would be, to a first approximation, a linear function of the reciprocal of their melting points. This would assume, of course, that the heat of fusion changes relatively slowly compared with $(T_m - T)$. That this general relation does, in fact, hold for the present situation is evident from Fig. 2.

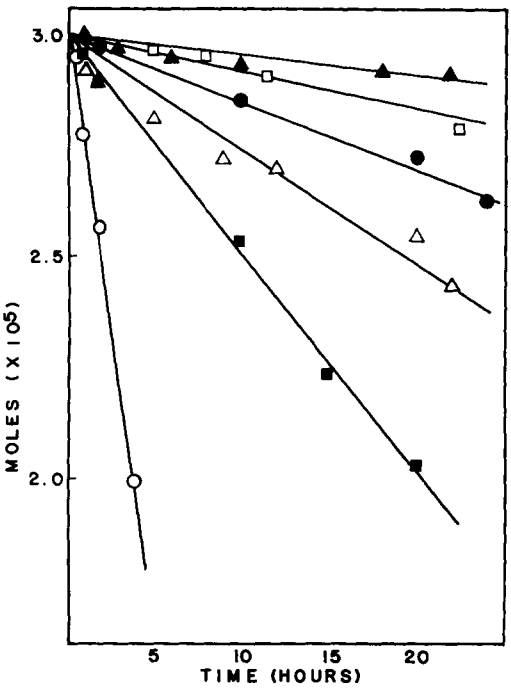


Fig. 1.—Solid state degradation of vitamin A compounds at 50°. ▲ Vitamin A benzhydrazone, m.p. 181–182°; □ vitamin A succinate triphenylguanidine salt, m.p. 140–140.5°; ●, vitamin A 3,4,5-trimethoxybenzoate, m.p. 85–86°; △ vitamin A nicotinate, m.p. 93–94°; ■ vitamin A phthalimido-N-acetate, m.p. 111–112°; ○ vitamin A acetate, m.p. 57–58°.

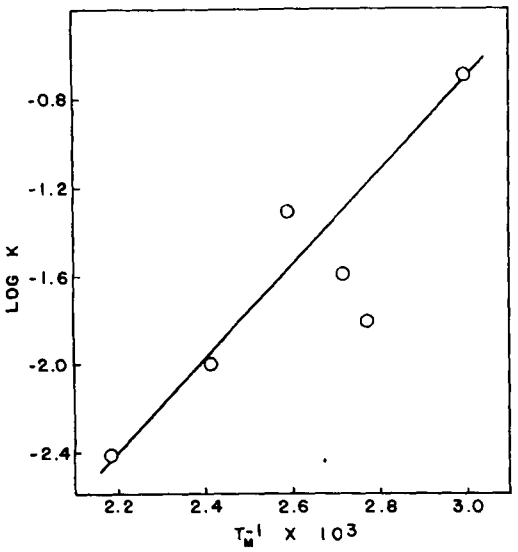


Fig. 2.—Relationship between zero-order rate constant and melting point for vitamin A derivatives.

From the slope of the line in this plot, an approximate mean value of 10 Kcal./mole is obtained for the compounds employed in this study. The heats of fusion of these vitamin A derivatives have not been determined experimentally, but they can be expected to be in the range of 10–18 Kcal./mole by comparison with similar compounds. This is in reasonable agreement with the mean value indicated by the slope and, therefore, supports the general line of reasoning presented above.

All points obviously cannot be expected to fall consistently on the average line in plots such as that shown in Fig. 2. Indeed, the effects of such uncontrolled factors as particle size, relative purity, and presence of catalytic species would also strongly influence the relative rates of degradation. The nature of the derivatives certainly must have some effect. It would appear, for example, that the greater stability of vitamin A 3,4,5-trimethoxybenzoate may be related to the fact that the acyl portion of the molecule may impart some free radical scavenging property to the whole molecule. This is suggested by the behavior of the ester in solution. The incorporation of even a mild antioxidant into the vitamin A molecule should increase its stability considerably.

None of the derivatives tested showed an appreciable induction period before degradation. Induction periods are often observed in reactions which proceed by free radical mechanisms. In such systems, nucleation is often a slow process, and some time elapses before foci of initiation reactions are developed. Many free radical processes show no induction period however, due to the reactivity of the molecules, involved.

Relative Stability of the Derivatives in Solution

The solution stabilities of the several vitamin A derivatives would not be expected to differ appreciably, particularly if the dissimilar portions of the molecule do not participate significantly in any free radical reactions. This is borne out in the data presented in Fig. 3.

These results were obtained in chloroform solution at room temperature. Measurements were carried out on solutions of essentially the same molar concentration since polymerization reactions have been shown to be approximately 1.5 order with respect to vitamin A (16). The pertinent measurements were limited to the initial stages of degradation just as they were in the case of the solid state study.

In contrast to its behavior in the solid state, vitamin A acetate appears to exhibit stability in solution comparable to that of other esters. The stabilizing effect of the strong lattice, which is apparently an important factor in determining the relative stability of the compounds in the solid state is, of course, no longer effective in solution. In fact, five of the compounds studied showed almost parallel decomposition rates. This finding appears to support the contention that the stability of compounds in the solid state is directly related to the strength of the crystal lattice.

The unusual stability of vitamin A benzhydrazone in solution is probably a reflection of its different structure. This difference in structure was not important in the solid state studies, where the principal degradative routes are probably autoxidation and polymerization; but in solution it becomes a determining factor in stability.

In solution, two reactions, in addition to autoxidation and polymerization, appear to participate in the degradation of vitamin A compounds. Isomerization of the polyolefin and formation of anhydro-

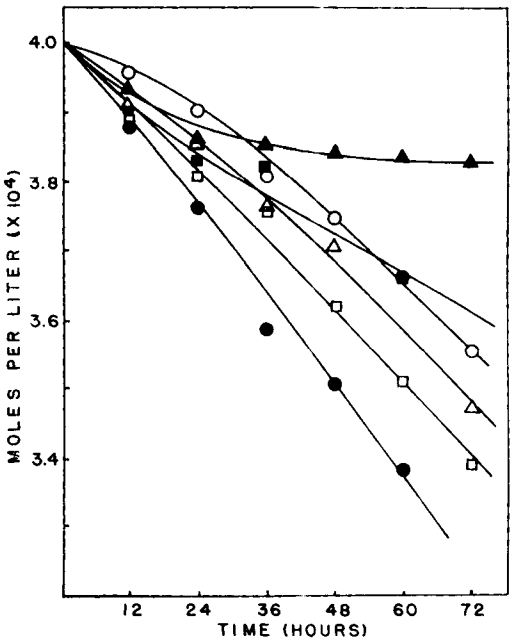


Fig. 3.—Degradation of vitamin A compounds in chloroform at room temperature. ▲ vitamin A benzhydrazone; ■ vitamin A nicotinate; O vitamin A 3,4,5-trimethoxybenzoate; △, vitamin A acetate; □ vitamin A phthalimido-N-acetate; ● vitamin A succinate triphenylguanidine salt.

vitamin A are indicated in the ultraviolet spectra of partially degraded samples. Both reactions are associated with the double bond system of the molecule. Vitamin A benzhydrazone has an additional double bond in conjugation with the double bonds in the vitamin A carbon chain. This additional double bond probably greatly influences the reactivity of the compound to isomerization and anhydrovitamin A formation.

PHARMACEUTICAL SIGNIFICANCE

It is likely, on the basis of present findings, that in a series of vitamin A derivatives, the compound having the highest melting point will be the most stable, all other factors being equal. Stability is not, however, the only criterion employed in selecting a vitamin A derivative for use in solid formulations. Equally important to the manufacturer and user of vitamin A products is the biological activity, or "availability," of the compound. Biological availability of many compounds is determined by the solubility of the substances in water and in fats and oils.

The same equation which was used to describe the influence of the melting point of a compound on its stability in the solid state can be used to correlate solubility with melting point. From this equation it is evident that for compounds having similar molar heats of fusion, those with lower melting points will have the higher solubilities. Thus, if all other factors are equal, compounds with high melting points will have lower solubilities, and hence, frequently lower biological availabilities.

The conclusions reached in this study with regard to vitamin A may also be applied to other unstable compounds. Pharmaceutical chemists have applied their energies toward the goal of increasing the solubility of compounds and of improving their taste by chemical modification. The suggested approach of using chemical alteration to provide derivatives having high melting points and hence, greater stability, should also prove useful in those instances where drug degradation poses a serious problem.

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Discoloration of Tablets Containing Amines and Lactose

By ROBERT A. CASTELLO and ALBERT M. MATTOCKS

It has been noted by several workers that tablets of amine salts combined with lactose darken in color with aging, and this problem has been investigated. Results show that the darkening is due to a reaction occurring in the presence of amine base and not in the presence of the salt. The reaction in tablets can be accelerated with elevated temperature and high humidity. The release of free base from the amine salt is shown to be due to reaction of the alkaline lubricant, such as magnesium stearate, which removes hydrogen ion with the formation of stearic acid, furnishing an alkaline medium in the adsorbed moisture. The change in pH with time was recorded to illustrate the effects occurring in tablets. The use of neutral or acidic lubricants, such as glyceryl monostearate or stearic acid, prevents the discoloration from occurring. These findings are thought to be applicable to any combination of amine salt and lactose.

THOUGH lactose has long been used as a tablet excipient, the recent finding that spray-dried lactose can be compressed without granulation has greatly increased the use of this material in tablet-making. This has made more important the recognition and avoidance of incompatibilities of lactose, combinations with amines being, perhaps, the one most commonly encountered.

A number of workers have found that tablets of amine salts combined with lactose as a filler have discolored slowly on storage to become tan in color. The intensity of color generally has been dependent on the amount of amine present and the humidity and time of storage, 6 months often being required for the discoloration to be distinct. It is of concern to learn whether the reaction occurring involves decomposition of the active ingredients, the lactose, or both, and how this reaction may be prevented. For these purposes the work presented in this paper was undertaken. Amphetamine sulfate was selected as the amine salt for most of this work.

EXPERIMENTAL

Materials Used.—Spray-dried lactose,[1] amphetamine sulfate, and various lubricants and excipients used were of U.S.P., N.F., or pharmaceutical grade. All chemicals used were of reagent grade.

A series of tablets were prepared of lactose with a relatively high percentage of amphetamine sulfate and with various lubricants, the combinations being selected so as to determine the ingredients necessary for reaction. Tablets were placed on watch glasses in a humidity cabinet at 40° and 85% relative humidity, conditions previously found to cause distinct discoloration in a commercial tablet in 1–2 days. Results are given in Table I.

Received April 28, 1961 from the College of Pharmacy, University of Michigan, Ann Arbor.
Accepted for publication June 30, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

[1] Western Condensing Co., Appleton, Wis.

These results indicated several possibilities for reaction: (*a*) the free amine may be necessary for reaction, in which case the reaction may occur between the amine and lactose, or the free base may simply be undergoing alkaline oxidation, (*b*) the reaction may be a general base-catalyzed reaction of lactose.

To demonstrate clearly the alkalizing effects of the lubricants, excess amounts of lubricant (3%) were added to a 1% solution of amphetamine sulfate, the mixture was stirred continuously, and the pH was recorded over a period of several hours. Curves of pH *vs.* time are shown in Fig. 1.

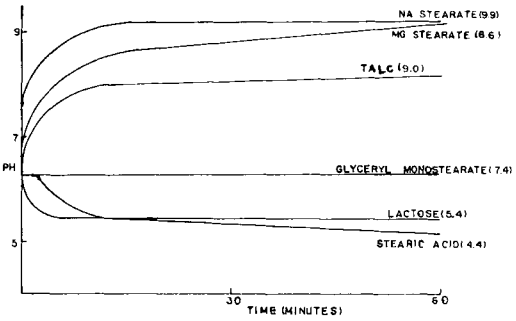


Fig. 1.—Effect of lubricants on solutions of amphetamine sulfate. Figures in parentheses are pH of suspensions of lubricants alone.

From Fig. 1 it is seen that the substances promoting discoloration, sodium stearate, magnesium stearate, and talc shifted the pH of the solution from 6.3 to values well on the alkaline side.

Results to this point indicated that the discoloration reaction required moisture, and it was concluded that the possibility of occurrence of reaction could be predicted from results with solutions. The use of solutions allowed observations to be made more quickly with discoloration being more distinct.

Results obtained with a series of solutions are shown in Table II. These data demonstrate that if an amine base or other alkali is present, the discoloration occurs. Further, it is seen that discoloration occurs only in the presence of lactose. Thus, it is clear that the discoloration is a base-catalyzed reaction of lactose.

TABLE I.—DISCOLORATION OF AMINE-LACTOSE TABLETS AFTER 48 HOURS AT 40° AND 85% RELATIVE HUMIDITY

Amine	Lubricant	Visual Observation
Amphetamine SO_4 10%	Mg Stearate 1%	Discoloration
Amphetamine SO_4 10%	Na Stearate 1%	Discoloration
Amphetamine SO_4 10%	Talc 2%	Discoloration
Amphetamine SO_4 10%	Stearic acid 1%	No discoloration
Amphetamine SO_4 10%	Glyceryl monostearate 1%	No discoloration
Amphetamine SO_4 10%	None	No discoloration
Amphetamine base 10%	None	Strong discoloration

TABLE II.—DISCOLORATION OF AQUEOUS SOLUTIONS AFTER 48 HOURS AT 60°

	Components		
Amine	Lactose, %	Other	Visual Observations
Amphetamine SO_4	10	...	No discoloration
.....	10	...	No discoloration
Amphetamine base, 1%	..	...	No discoloration
Amphetamine base, 1%	10	...	Severe discoloration
Ammonia buffer pH 9.5	10	...	Slight discoloration
.....	10	NaOH, 1%	Severe discoloration
.....	10	Mg stearate, 3%	No discoloration
Amphetamine SO_4, 1%	10	Mg stearate, 3%	Severe discoloration
Desoxyephedrine HCl, 1%	10	...	No discoloration
Desoxyephedrine base, 1%	10	...	Severe discoloration
Desoxyephedrine HCl, 1%	10	Mg stearate, 3%	Severe discoloration

The fact that the reaction product is from lactose alone and does not contain amine was substantiated by extraction and examination of the colored material. A solution of lactose, 10%, and amphetamine base, 1%, was allowed to react at 60° for several days, and the colored material was extracted with *n*-butanol. The butanol solution was washed thoroughly with water, dilute hydrochloric acid, and again with water, little or none of the color being removed in the washings. The butanol solution was then dried over anhydrous sodium sulfate and evaporated to leave a dark, tarry residue of the colored product. This material could not be recrystallized but was found to be free of nitrogen. Treatment with phenylhydrazine yielded yellowish crystals, recrystallized from methanol-water, which melted at 186–188°. This derivative has not yet been identified.

DISCUSSION

Reactions of carbohydrates with amines are well known and have been reviewed by Pigman (1). It has been found that reactions occur with aldehyde groups of open chain forms of the carbohydrates to form Schiff bases, as well as with ring forms to produce glycosylamines or N-glycosides. The reaction causing formation of a colored product, however, is more likely the browning reaction described by Maillard in 1912 (2). Beacham and Dull (3) investigated the browning of glucose, 2,3,4,6-tetramethyl-D-glucose, glyceric aldehyde, glycoaldehyde, and a number of glycoaldehyde ethers in the presence of amines. They observed that the susceptibility to browning of the D-glycosyl derivatives of the various amines was directly proportional to the basic strength of the amine. From the work of Mitts and Hixon (4) it is seen that the glucosyl derivatives of the more basic amines were most highly dissociated in aqueous solution, while those amines which formed condensation products resistant to hydrolysis did not readily cause browning. The amine derivatives of glyceric aldehyde and glycoaldehyde browned readily, indicating that furfurals are not necessary intermediates in the browning reaction. Derivatives formed from etherified glycoaldehyde and 2,3,4,6-tetramethylglucose were resistant to browning, supporting the necessity of the α-hydroxyl group in the reaction. This was in agreement with Hurd and Kelso (5) who reported that 2,3,4-tridesoxyaldopentose did not brown in the presence of glycine while 3,4-didesoxyaldopentose did. Results of these workers indicate that although a reaction of sugar with amine may be an intermediate step, it is not a necessary step in the browning reaction.

The reaction occurring between the amine salt and the basic lubricants, sodium stearate for example, may be postulated as

$$RN^+H_3 = RNH_2 + H^+ \qquad \text{(Eq. 1)}$$

$$\text{Stearate}^- + H^+ = \text{HStearate} \qquad \text{(Eq. 2)}$$

$$RN^+H_3 + \text{Stearate}^- = RNH_2 + \text{HStearate} \qquad \text{(Eq. 3)}$$

In the reaction mixture excess stearate is available; therefore, the alkali stearate is maintained at saturation. The reaction causes the solution to become saturated with stearic acid, as well, providing sufficient hydrogen ion is released from the amine salt. The equilibrium pH of the mixture may be approximated by the Henderson-Hasselbalch equation

$$\text{pH} = \text{pKa}_{(\text{HStearate})} + \log\left(\frac{\text{Stearate}^-}{\text{HStearate}}\right) \qquad \text{(Eq. 4)}$$

The theoretical value calculated by Eq. 4 was 8.49, which does not agree with the observed value of 9.2 for the sodium stearate mixture (see Fig. 1). It was felt, therefore, that either the proposed mechanism was incorrect or the pH measurements of the mixture were in error. It was noted that Jenny, *et al.* (6), reported the development of a liquid junction potential when measuring the pH of slurries and colloidal systems. This potential was said to be due

to a change in transference numbers of the potassium and chloride ions in the solution being measured. They demonstrated this effect by observing a potential drop across two calomel electrodes, one immersed in the suspension, the other in the supernatant liquid.

To eliminate this error, an equilibrium mixture of amphetamine sulfate and sodium stearate was centrifuged to remove the suspended material. The pH was again determined and found to be 8.6, which is in reasonable agreement with the calculated value, 8.49, from Eq. 4.

From these results it is proposed that Eq. 3 is descriptive of the system, and that the role of basic lubricants is the removal of hydrogen ion to promote the base-catalyzed browning of lactose. These findings coupled with later tests using inorganic alkalies indicate that the reaction is a general base-catalyzed reaction and not limited to amine bases.

It should be noted that the pH values shown in Fig. 1 are those of suspensions and contain the errors discussed above.

Results of this work furnish a practical means for predicting discoloration of tablets containing lactose with a few short-term tests. It is suggested that a new formulation be tested simply by preparing a solution or slurry of the ingredients, placing it in a bath at 60° for 2 or 3 days, and then observing the color. In this way one can determine whether the formulation is satisfactory before preparing tablets. Also, it is demonstrated that lactose should not be utilized in combination with bases, and when lactose is combined with amine salts, alkali stearates and talc should be avoided.

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Synthesis of α- and β-Amino Ketone Analogs of Amino Acids as Antibacterial Agents

By SHU-SING CHENG†, SIGURDUR JONSSON‡, and FRED T. SEMENIUK

α- and β-Amino ketone analogs of amino acids were synthesized as potential antagonists of amino acids in microbial metabolism. The hypothetical mechanism of antibacterial activity for certain analogs is proposed. The successful use of organocadmium reagents for the synthesis of the aliphatic and aromatic series of α- as well as β-amino ketones indicates a wider scope of applicability than by the method of Dakin-West or by Friedel-Crafts acylation. These series of compounds show antimicrobial activity *in vitro*.

THE ANTIBACTERIAL activity (1) reported for β-amino ketone analogs of β-alanine suggested that α-amino ketone analogs of α-amino acids might possess similar activity. Due to the structural similarity between amino ketone analogs and the natural amino acids, one may assume that these analogs function as antagonists to amino acids, thus interfering with biochemical reactions in which individual amino acids or related peptides participate.

The cell walls of most Gram-positive bacteria consist primarily of components linked together as the following sequence: 3-O-carboxyethyl-hexosamine-*dl*-alanine-*d*-glutamic acid *l*-lysine-*dl*-alanine-*dl*-alanine (2). It could be assumed that an amino ketone analog of α-alanine would interfere with the formation of this peculiar amino-sugar peptide, thus inhibiting the cell wall synthesis. Many species of bacteria are able to grow on simple media containing ammonium and other inorganic salts, plus a single organic carbon source such as glucose. Some species require the addition of certain amino acids, simple peptides, and vitamins for growth. It is obvious that many bacteria can synthesize a majority of amino acids from ammonium salt and glucose. Two ways are recognized by which a bacterium can assimilate ammonia and convert it to α-amino groups in amino acids. These are the fumarate and the α-ketoglutarate pathways (3). The fumarate pathway is dependent upon a condensation reaction between fumaric acid and ammonia to yield aspartic acid. The α-ketoglutarate pathway is

Received April 28, 1961, from the School of Pharmacy, University of North Carolina, Chapel Hill.
Accepted for publication May 8, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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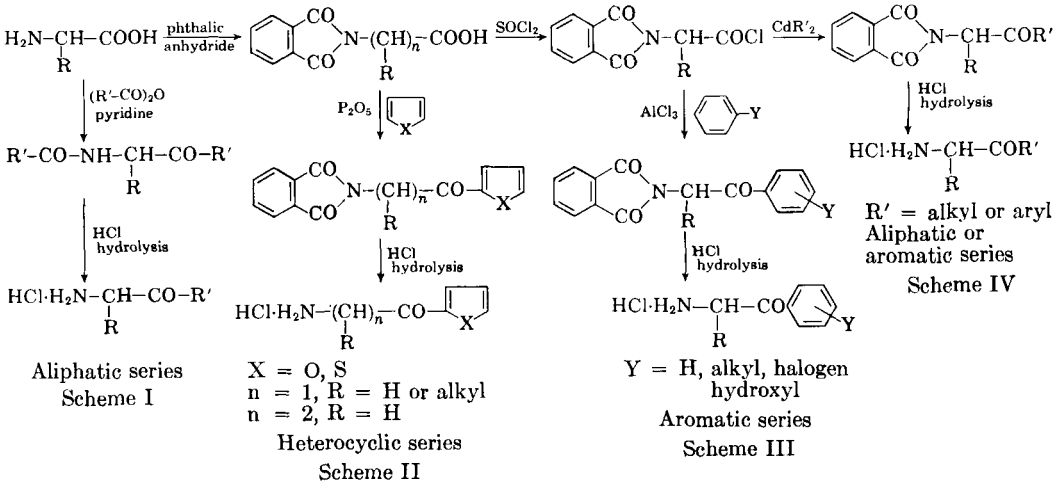
By SHU-SING CHENG†, SIGURDUR JONSSON‡, and FRED T. SEMENIUK

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‡ Deceased.



based upon a reaction involving α-ketoglutarate and ammonia, the product being *l*-glutamic acid. Both aspartic and glutamic acids can serve as an amino group "pool" from which various amino acids are derived through transamination reaction between the pool and a variety of α-keto acids under the catalysis of transaminases. It follows that amino ketone analogs of aspartic acid or glutamic acid would block the formation of most amino acids.

Glycine combines with cysteine and glutamic acid to yield a tripeptide, γ-glutamylcysteinylglycine (glutathione). Glutathione functions as an important prosthetic group in the enzyme glyceraldehyde-3-phosphate dehydrogenase (4) which catalyzes the dehydrogenation and phosphorylysis of glyceraldehyde-3-phosphate, thus converting it to 1,3-diphosphoglyceric acid in the anaerobic breakdown of glucose (5). The amino ketone analogs, H_2NCH_2COR, of glycine conceivably might condense with cystein and glutamic acid to form a pseudo tripeptide, $HOOCCH(NH_2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COR$, with a ketone fragment instead of a carboxyl group at one end of the peptide chain. This analog of glutathione might compete with glutathione for the enzyme glyceraldehyde-3-phosphate dehydrogenase. In this fashion, the conversion of glyceraldehyde-3-phosphate to 1,3-diphospho-glyceric acid is preventable, and the microbial utilization of glucose may be interrupted. Phenylalanine, tyrosine, and leucine have been shown to be essential amino acids for the growth of psittacosis virus in chick embryo tissue (6). Presumably, amino ketone analogs of these amino acids might inhibit the growth of this virus. In addition to the proposed hypothetical mechanism for the antibacterial activity of each particular amino acid, the amino ketone analogs might interfere with peptide formation in protein synthesis in general.

Most of the aliphatic and aromatic amino ketone analogs of glycine, α-alanine, leucine, phenylalanine, tyrosine, and aspartic acid were synthesized by the hydrolysis of acylamido or phthalimido ketones obtained by the Dakin-West method or by Friedel-Crafts acylation, respectively. The heterocyclic β-aminoethyl ke-

TABLE I.—α-ACYLAMIDO KETONES

$$R{-}CH(NH{-}CO{-}CH_2CH_2CH_3){-}CO{-}CH_2CH_2CH_3$$

R	M.P., °C.	Yield, %	Formula	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
$CH_3{-}CH(CH_3){-}CH_2{-}$	b.p. 265 $n^{29°}$ 1.4618	35	$C_{13}H_{25}NO_2$	68.9	69.0	11.1	10.98	6.17	6.70
$C_6H_5{-}CH_2{-}$	63–64	28	$C_{16}H_{23}NO_2$	73.5	74.0	8.85	8.73	5.37	5.25
$p{-}HO{-}C_6H_4{-}CH_2{-}$	108–109	39	$C_{16}H_{23}NO_3$	70.8	69.8	8.35	8.64	5.05	4.88

[a] Analyses by Weiler and Strauss, Oxford, England.

TABLE II —α-PHTHALIMIDO KETONES

R_1	R_2	M.P., °C.	Yield, %	Formula	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Cl or Br, % Calcd.	Cl or Br, % Found
H	*p*-Cl—C_6H_4—	216–217	61	$C_{16}H_{10}ClNO_3$	64.05	63.82	3.37	3.28	4.67	4.67	11.81	11.68
H	*p*-Br—C_6H_4—	227–228	33	$C_{16}H_{10}BrNO_3$	55.9	56.0	2.95	3.08	4.08	3.69	23.1	22.8
H	*n*-C_7H_{15}—	56–57		$C_{17}H_{21}NO_3$	71.00	70.85	7.34	7.41	4.86	4.69		
H	*p*-CH_3—C_6H_4—	186–187	18	$C_{17}H_{13}NO_3$	73.09	72.70	4.69	4.88	5.02	5.07		
			32									
H	*o*-CH_3—C_6H_4—	130–131	14	$C_{17}H_{13}NO_3$	73.09	73.30	4.69	4.62	5.02	4.82		
H	3,4-diCH_3—C_6H_3—	165–166	70	$C_{18}H_{15}NO_3$	73.69	74.0	5.16	5.11	4.78	4.54		
H	C_6H_5—	167–168	84	$C_{16}H_{11}NO_3$	72.43	73.20	4.15	4.11	5.30	5.10		
CH_3—	C_6H_5—	87–88	85	$C_{17}H_{13}NO_3$	73.0	73.46	4.66	4.91	5.02	4.93		
CH_3—	*p*-CH_3—C_6H_4—	110–111	53	$C_{18}H_{15}NO_3$	73.69	73.78	5.16	5.24	4.78	4.84		
CH_3—	3,4-diCH_3—C_6H_3—	114–115	57	$C_{19}H_{17}NO_3$	74.01	75.0	5.58	5.53	4.56	4.26		
$HOOCCH_2$—	C_6H_5—	220–221		$C_{18}H_{13}NO_5$	67.0	66.66	4.05	4.22	4.34	4.22		
$HOOCCH_2$—	*p*-CH_3—C_6H_4—	187–188		$C_{19}H_{15}NO_5$	67.7	67.14	4.47	4.85	4.15	4.20		
$C_6H_5CH_2$—	*n*-C_6H_{13}—	86–87	15	$C_{23}H_{25}NO_3$	76.2	75.96	6.95	6.58	3.85	3.80		

[a] Analyses by Weiler and Strauss, Oxford, England.

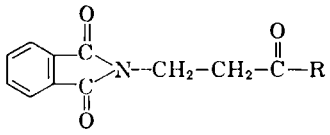
tone analogs of β-alanine were synthesized by the hydrolysis of phthalimido ketones obtained from the condensation of thiophene or furan and β-phthalimido-propionic acid under the catalysis of phosphorus pentoxide. These heterocyclic analogs have not been reported previously.

The adaptation of organocadmium reagent by Cheng and Jonsson (1) for the preparation of alkyl β-aminoethyl ketones proved feasible. It therefore seemed of some interest to study the possibility of utilizing this reagent for the preparation of α-amino ketone analogs of amino acids.

The above mentioned amino ketone analogs were prepared as summarized by the reaction schemes. In scheme I, the aliphatic series of α-amino ketones are prepared by the hydrolysis of acylamido ketones, obtained conveniently by heating an α-amino acid with an aliphatic acid anhydride in pyridine according to Dakin and West (7). Since excessive amounts of acid anhydride are required for the formation of the acylamido ketone, the high cost of the higher member acid anhydride would render the preparation of acylamido ketone impractical. Furthermore, alkyl aminomethyl ketones are not attainable by the treatment of glycine with acid anhydride in pyridine. Glycine is subject to decomposition by the action of acetic anhydride and its higher homologs after a short period of heating in pyridine. These difficulties encountered in the synthesis of amino ketone analogs of α-amino acids by the Dakin-West method were overcome by the use of organocadmium reagent as illustrated in scheme IV.

In scheme III, the aromatic series of α-amino ketones are prepared by the hydrolysis of phthalimido ketones obtained from the condensation of phthalimidoacyl chlorides and aromatic hydrocarbons under the catalysis of anhydrous aluminum chloride according to Friedel-Crafts acylation reaction. The yields range from 60 to 80%. Unfortunately this reaction failed to yield β-phenyl α-phthalimidopropiophenone when β-phenyl α-phthalimidopropionyl chloride was treated with anhydrous aluminum chloride in dry benzene, 2-phthalimido-1-indanone being obtained instead. This unexpected compound was formed from the intramolecular acylation of the β-phenyl nucleus by the acyl carbonyl of the same molecule. The tendency of five-membered ring closure was so pronounced that the competitive intermolecular acylation reaction was overwhelmed, so that the expected product was not formed. A study of the factors which govern the pattern of the reactions reveals the operation of the inductive as well as of the

TABLE III.—β-PHTHALIMIDOETHYL KETONES



R	M.P., °C.	Yield,[a] %	Formula	Analyses[b] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	S, % Calcd.	S, % Found
	148	35	$C_{15}H_{11}NO_4$	66.88	67.50	4.13	4.23	5.21	5.45		
	154–155	38	$C_{15}H_{11}NO_3S$	62.30	63.30	5.22	4.72	4.84	4.85	11.08	10.88

[a] Based on the quantity of β-phthalimidopropionic acid consumed. [b] Analyses by Weiler and Strauss, Oxford, England.

mesomeric effects. The difficulty however, in obtaining the desired intermediate for the preparation of β-phenyl-α-aminopropiophenone was overcome by the use of organocadmium reagent as outlined in scheme IV, where R is a benzyl and R′ phenyl, respectively.

The successful preparation of aliphatic and aromatic series of α-amino ketone analogs of amino acids by the use of organocadmium reagent demonstrates the wide scope of applicability and the advantage of this method over that of the Dakin-West method and of Friedel-Crafts acylation. 1-Phenyl-2-phthalimido-3-nonanone was prepared in a yield of 15%, comparable with 14–20% for the alkyl β-phthalimidoethyl ketones prepared from β-phthalimidopropionyl chloride by the same method (1). This reveals that the bulky α-phthalimido as well as the β-phenyl group exerts no steric hindrance on the condensation of the phthalimidoacyl chloride with either the alkyl or aryl organocadmium reagent.

The isolation of biphenyl from a reaction using phenylcadmium for the preparation of β-phenyl-α-phthalimidopropiophenone reveals the decomposition of organocadmium reagent in the course of reaction. This probably accounts for the low yield of amino ketone prepared by this method. The probable formation of the aliphatic hydrocarbons has not been demonstrated, perhaps due to the high volatility of the alkanes. The overall yield of amino ketones prepared by this method ranges from 20 to 40%, whereas it ranges from 32 to 34%, and 40 to 50% by Dakin-West and Friedel-Crafts methods, respectively.

These series of α- and β-amino ketones have been found to be active *in vitro* against the growth of bacteria and fungi when tested by the serial broth dilution and agar diffusion methods. The results of *in vitro* screening will appear in a subsequent paper.

EXPERIMENTAL

The chemical procedures used for the preparation of the various intermediates and products are illustrated by the following examples:

Phthalimidocarboxylic Acids.—Phthalimidoacetic, α-phthalimidopropionic, β-phthalimidopropionic, β-phenyl-α-phthalimidopropionic, and phthaloylaspartic acids were prepared according to Gabriel's method (8), with modification in the process of purification as described for β-phenyl-α-phthalimidopropionic acid: 100 Gm. of *dl*-phenylalanine (0.60 mole) and 90 Gm. phthalic anhydride (0.60 mole) were melted together at 210°, and the melt was maintained at this temperature for one-half hour. The product was cooled to room temperature and then dissolved in 150 ml. alcohol by refluxing. White crystalline powder, 165 Gm., was obtained after the solution was cooled to room temperature, corresponding to a yield of 82%. Recrystallization from alcohol gave large rhombic crystals, m.p. 177–178°.

Phthalimidoacyl Chlorides.—Phthalimidoacetyl, α-phthalimidopropionyl, β-phenyl-α-phthalimidopropionyl, and phthaloylaspartyl chlorides were prepared by the procedure described for β-phenyl-α-phthalimidopropionyl chloride: a mixture of 110 Gm. β-phenyl-α-phthalimidopropionic acid (0.5 mole), and 148 Gm. thionyl chloride (1.24 moles) was refluxed until all the solid dissolved. The excess thionyl chloride (25 ml.) was recovered by distillation under reduced pressure. The dark brown residue from the distillation was repeatedly extracted with petroleum ether (30–60°) by refluxing. A 102-Gm. quantity of chloride was obtained, corresponding to a yield of 85%, m.p. 73°.

Aliphatic Series of Acylamido Ketones. (See Table I).—4-Butyramido-2-methyl-5-octanone, 1-phenyl-2-butyramido-3-hexanone, 1-(*p*-hydroxy)phenyl-2-butyramido-3-hexanone were prepared by the procedure described for 1-(*p*-hydroxy)phenyl-2-butyramido-3-hexanone: a mixture of 10 Gm. of *dl*-tyrosine (0.055 mole), 300 ml. pyridine, and 43 ml. of *n*-butyric anhydride was refluxed for 8 hours. The excessive *n*-butyric anhydride and pyridine were removed by distillation under reduced pressure. The residual liquid was poured, with stirring, into 400 ml. petroleum ether (b.p. 30–60°). Six grams of 1-(*p*-hydroxy)phenyl-2-butyramido-3-hexanone was isolated, corresponding to a yield of 39%. It melted

TABLE IV.—α- AND β-AMINO KETONE HYDROCHLORIDES

$$HCl.H_2N-\underset{\displaystyle R_1}{\underset{|}{(CH)_n}}-CO-R_2$$

n	R_1	R_2	M.P., °C.	Yield, %	Formula[b]	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Cl, % Calcd.	Cl, % Found
1	H	C_6H_5—	186[c]	78	C_8H_9NO	56.0	55.8	5.85	5.77	8.18	8.29		
1	H	p-$CH_3C_6H_4$—	206[c]	82	$C_9H_{12}NO$	58.2	57.6	6.51	6.44	7.55	7.38		
1	H	o-$CH_3C_6H_4$—	160[c]	76	$C_9H_{12}NO$	58.2	57.4	6.51	6.77	7.55	7.15		
1	H	3,4-di$CH_3C_6H_3$—	213[c]	76	$C_{10}H_{14}NO$	60.2	60.13	7.03	7.28	7.01	7.3	17.7[f]	17.2
1	H	p-ClC_6H_4—	279–280[c]	69[d]	C_8H_8ClNO	46.5	47.1	4.36	4.42	6.81	6.82	34.5[g]	35.0
1	H	p-BrC_6H_4—	285[c]	74[d]	C_8H_8BrNO	38.5	39.0	3.63	3.80	5.60	5.80		
1	H	n-C_7H_{15}—	157–159	51	$C_9H_{19}NO$	55.85	55.0	10.4	10.1	7.24	7.42	18.25[f]	18.80
1	CH_3—CH(CH_3)—CH_2—	n-C_3H_7	89–90	62	$C_9H_{19}NO$	55.95	56.08	10.4	9.99	7.24	6.83		
1	CH_3	C_6H_5—	183–184[c]	48	$C_9H_{11}NO$	58.2	57.7	6.49	6.90	7.56	7.70		
1	CH_3	p-$CH_3C_6H_4$—	232[c]	63	$C_{10}H_{13}NO$	60.1	59.88	7.09	7.15	7.01	7.24		
1	CH_3	p-HOC_6H_4—	206–208[c]	33	$C_9H_{11}NO_2$	53.80	53.85	6.01	6.48	6.96	6.50		
1	CH_3	3,4-di$CH_3C_6H_3$—	180[c]	14	$C_{11}H_{15}NO$	61.9	61.5	7.55	7.71	6.58	6.32		
1	$C_6H_5CH_2$—	n-C_3H_7—	127–128	43	$C_{12}H_{17}NO$	63.2	62.36	7.93	8.03	6.14	6.18	15.59	15.95
1	$C_6H_5CH_2$—	n-C_6H_{13}—	108–109	27	$C_{15}H_{23}NO$	66.95	66.10	8.97	8.61	5.20	5.40	13.4	14.0
1	$C_6H_5CH_2$—	C_6H_5—	215–216[c]	22[e]	$C_{15}H_{15}NO$	68.9	68.23	6.18	6.13	5.35	5.35	13.59	13.95
1	$C_6H_5CH_2$—	p-$CH_3C_6H_4$—	207[c]	14[e]	$C_{16}H_{17}NO$	69.7	68.65	6.58	6.44	5.26	4.95	12.81	13.20
1	$HOOCCH_2$—	C_6H_5—	204[c]	71	$C_{10}H_{11}NO_3$	52.30	52.27	5.28	5.34	6.10	6.52		
1	$HOOCCH_2$—	p-$CH_3C_6H_4$—	198–199[c]	28	$C_{11}H_{13}NO_3$	54.40	54.38	5.80	5.74	5.76	5.59		
1	p-$HOC_6H_4CH_2$—	n-C_3H_7	147–148	22	$C_{12}H_{17}NO_2$	59.30	60.20	7.45	7.22	5.75	6.03		
2	H		173–174[c]	49	$C_7H_9NO_2$	48.00	48.10	5.76	5.63	8.06	8.00		
2	H		160–161	62	C_7H_9NOS	44.00	44.15	5.28	5.47	7.32	7.90	S, % 16.79	17.00

[a] Calculated as hydrochloride. [b] Formula of free amino ketone. [c] Melts with decomposition. [d] Yield based on the quantity of phthalimido ketone consumed. [e] Overall yield based on the quantity of phthalimidoacyl chloride used. [f] Cl% of the hydrochloride. [g] Cl%, including organic-bound Cl, of the hydrochloride.

at 108–109° after recrystallization from a mixture of benzene and petroleum ether.

Alkyl and Aryl α-Phthalimido Ketones (See Table II).—The aryl phthalimido ketones were prepared by Friedel-Crafts acylation, excepting β-phenyl-α-phthalimidopropiophenone, not obtainable by Friedel-Crafts acylation, which was prepared by the condensation of β-phenyl-α-phthalimidopropionyl chloride and phenylcadmium according to the procedure (1) described for the synthesis of alkyl β-phthalimidoethyl ketones. The procedure for the alkyl and aryl α-phthalimido ketones is essentially the same as that for the β-phthalimidoethyl ketones, except for the process of purification, as described for 1-phenyl-2-phthalimido-3-nonanone: the organocadmium reagent was prepared from 36.6 Gm. of *n*-hexyl bromide (0.22 mole), 5.3 Gm. magnesium turnings (0.22 mole), and 16 3 Gm. anhydrous cadmium chloride (0.22 mole) in anhydrous ether. After the solvent was replaced with dry benzene, 42 Gm. β-phenyl-α-phthalimidopropionyl chloride (0.134 mole) in benzene was added to the mixture. The reaction was completed and the product isolated in the usual way. The thick oil obtained after the removal of benzene was distilled under reduced pressure, yielding a yellowish oil at 70–240°/0.5 mm. Crystals, 7.6 Gm., were obtained from the alcoholic solution of the oil, corresponding to a yield of 15%. The crystals melted at 86–87° after two recrystallizations from alcohol.

Heterocyclic β-Phthalimidoethyl Ketones (See Table III).—2-Furyl, and 2-thienyl β-phthalimidoethyl ketones were prepared by the procedure described for 2-thienyl β-phthalimidoethyl ketone: a mixture of 22 Gm. β-phthalimidopropionic acid (0.1 mole) and 100 ml. dry benzene was refluxed for 1 hour with constant stirring. The mixture was cooled to room temperature, and 10 Gm. phosphorus pentoxide was added in one portion. The mixture was stirred for 1 hour at room temperature and then refluxed for 5 hours, following which it was filtered, and the filtrate washed successively with 10% sodium carbonate and water, and dried over anhydrous sodium sulfate. A syrupy liquid, obtained when the solvent was removed from the benzene solution, crystallized as a white powder, weighing 6.25 Gm. and corresponding to a yield of 37.5% based upon the quantity of β-phthalimidopropionic acid actually consumed (12.3 Gm.). The white powder crystallized from boiling alcohol as short needles, m.p. 154–155°.

Amino Ketone Hydrochlorides (See Table IV).—Amino ketone hydrochlorides were prepared by the hydrolysis of the phthalimido or the acylamido ketones in a mixture of glacial acetic and concentrated hydrochloric acids under refluxing conditions according to the procedure described in the previous paper (1).

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Solvolysis of Symmetrical and Mixed Aspirin Anhydrides in 75% Dioxane–25% Water

By EDWARD R. GARRETT†

The neutral solvolysis of aspirin anhydride, I, and the mixed anhydrides of aspirin and acetic acid, II, of acetylsalicoylsalicylic and acetic acids, III, have been studied as functions of temperature in 75% dioxane by volume so as to compare them with the literature values on *m*- and *p*-substituted benzoic anhydrides studied in the same solvent. There is very little change in the heats of activation but large increases in the frequency factors of the *ortho*, mixed or symmetrical, over the *meta* and *para*. The mixed anhydrides of an *o*-acylbenzoic acid and acetic acid have higher solvolysis rates by virtue of lower frequency factors than the symmetrical aspirin anhydrides.

RECENT STUDIES (1) have demonstrated the presence of all possible products of acyl transfer in a melt of aspirin anhydride, I. Identification of these products necessitated the synthesis of several mixed anhydrides of aspirin and aspirin derivatives with acetic acid, *viz.*, the mixed anhydride of aspirin and acetic acid, II, and the mixed anhydride of acetylsalicoylsalicylic acid and acetic acid, III.

Received March 13, 1961, from the Research Division of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication May 24, 1961.
The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance.
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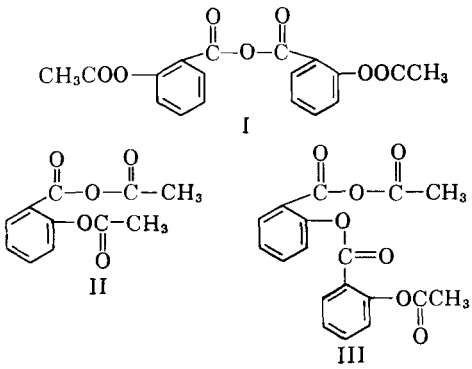
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By EDWARD R. GARRETT†

The neutral solvolysis of aspirin anhydride, I, and the mixed anhydrides of aspirin and acetic acid, II, of acetylsalicoylsalicylic and acetic acids, III, have been studied as functions of temperature in 75% dioxane by volume so as to compare them with the literature values on *m*- and *p*-substituted benzoic anhydrides studied in the same solvent. There is very little change in the heats of activation but large increases in the frequency factors of the *ortho*, mixed or symmetrical, over the *meta* and *para*. The mixed anhydrides of an *o*-acylbenzoic acid and acetic acid have higher solvolysis rates by virtue of lower frequency factors than the symmetrical aspirin anhydrides.

RECENT STUDIES (1) have demonstrated the presence of all possible products of acyl transfer in a melt of aspirin anhydride, I. Identification of these products necessitated the synthesis of several mixed anhydrides of aspirin and aspirin derivatives with acetic acid, *viz.*, the mixed anhydride of aspirin and acetic acid, II, and the mixed anhydride of acetylsalicoylsalicylic acid and acetic acid, III.

Received March 13, 1961, from the Research Division of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication May 24, 1961.
The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance.
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I

II

III

Since a significant amount of data has been obtained on the neutral hydrolysis of *m*- and *p*-substituted benzoic anhydrides in 75% dioxane-25% water solutions (2), studies on the symmetrical *ortho*-substituted benzoic anhydride, aspirin anhydride, and other unsymmetrical anhydrides of this type under the same conditions are of interest.

EXPERIMENTAL

Comparative Solvolysis Rates of Symmetrical and Nonsymmetrical Anhydrides in 75% Dioxane.—The general procedure for the hydrolysis studies in aqueous dioxane was to take a weighed amount of the anhydride up to a given volume with 75% dioxane. Prior to use, the dioxane was refluxed at least 24 hours over sodium to destroy peroxides, distilled, and the first fractions discarded. Both the dioxane and the distilled water used were thoroughly purged with nitrogen. The studies were made on the Cary model 11 recording spectrophotometer in 75% dioxane on aliquots of the solutions maintained in constant temperature baths.

The aspirin anhydride (3), I, aspirin, and salicylic acid were dried under high vacuum for several days before use.

The aspirin anhydride solutions were 2.142×10^{-4} *M*, the mixed anhydride of acetylsalicoylsalicylic acid and acetic acid, III, solutions were 3.827×10^{-4} *M*, and the solutions of the mixed anhydride of aspirin and acetic acid, II, were 6.274×10^{-4} *M*. The preparation of these latter compounds, II and III, has been previously described (1).

The ultraviolet spectra of these compounds are given in Fig. 1 and are plotted as the appropriate multiple of the molar absorptivity against wavelength in mμ since hydrolysis of one-half mole of aspirin anhydride would give one mole of aspirin and subsequently one mole of salicylic acid.

The high spectrophotometric purity of the mixed anhydrides was assured by the fact that the ultraviolet spectra of the hydrolyzing mixed anhydride of acetic acid and aspirin became the ultraviolet spectra of aspirin. The latter, as given in Fig. 1, had been determined from pure aspirin solution in 75% dioxane.

Salicylic acid production did not interfere with the determination of the rates of anhydride hydrolysis. For example, the rate constant for the hydrolysis of aspirin in 75% dioxane at 70.0° is 3.14×10^{-6} sec.$^{-1}$, which is 100 times slower than the hydrolysis of the mixed anhydride of aspirin and

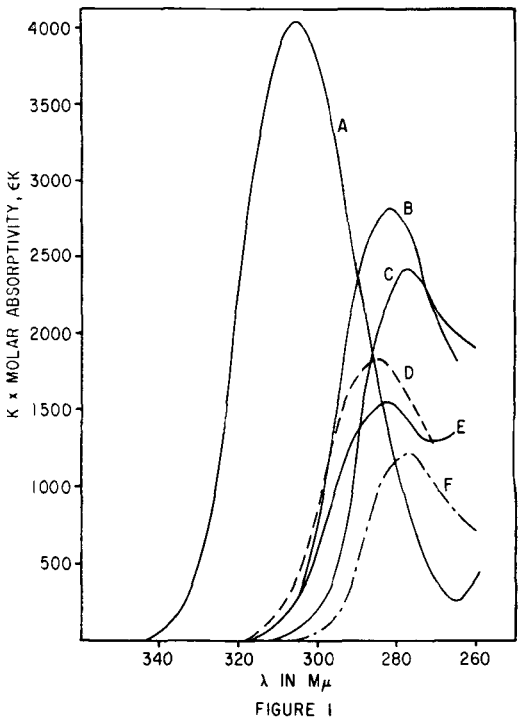


Fig. 1.—Ultraviolet spectra of anhydrides and their hydrolytic derivatives in 75% dioxane-25% water. *A*, Salicylic acid, $K = 1$; *B*, mixed anhydride, acetic and acetylsalicoylsalicylic acids, $K = 1$; *C*, acetylsalicoylsalicylic acid, $K = 1$; *D*, aspirin anhydride, $K = 1/2$; *E*, mixed anhydride, acetic acid, and aspirin, $K = 1$; *F*, aspirin, $K = 1$.

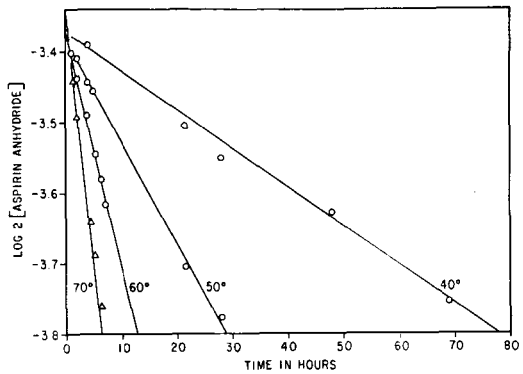


Fig. 2.—First-order plots of the hydrolysis of aspirin anhydride in 75% dioxane-25% water. Initial concentration 2.20×10^{-4} *M*.

acetic acid, II, and more than 12 times slower than the hydrolysis of aspirin anhydride, I, under the same conditions (see Table I).

However, since the spectra of the resultant highly chromophoric salicylic acid might interfere with the calculations for the aspirin anhydride to aspirin hydrolysis rates, the concentration of aspirin anhydride was calculated from the expression

$$[\text{Aspirin Anhydride}] = \frac{A'_{292.5}\,\epsilon^{ASP}_{277.5} - A'_{277.5}\,\epsilon^{ASP}_{292.5}}{\epsilon^{AA}_{292.5}\,\epsilon^{ASP}_{277.5} - \epsilon^{ASP}_{292.5}\,\epsilon^{AA}_{277.5}} \quad \text{(Eq. 1)}$$

TABLE I.—APPARENT FIRST-ORDER RATE CONSTANTS IN SEC.$^{-1}$ AND THERMODYNAMIC QUANTITIES FOR THE HYDROLYSIS OF VARIOUS ANHYDRIDES IN DIOXANE-WATER SOLUTIONS

	Aspirin Anhydride	Mixed Anhydride of Acetylsalicoylsalicylic Acid and Acetic Acid	Mixed Anhydride of Acetylsaliylic Acid and Acetic Acid
10^4 k at 27.0° in 10% dioxane	8.5[b]	62.0	24.0
10^4 k at 70.0° in 75% dioxane	0.474	5.18	5.62
10^4 k at 60.0° in 75% dioxane	0.216	2.08	2.81
10^4 k at 50.0° in 75% dioxane	0.0944	1.08	1.38
10^4 k at 40.0° in 75% dioxane	0.0351	0.52	0.78
S_a[a]	3,890	3,503	3,413
ΔH_a(Kcal./mole)	17.8	16.0	15.6
log P[a]	7.02	6.89	6.70

[a] The quantities are derived from the logarithmic form of the Arrhenius relation: $\log k = -(\Delta H_a/2.303R)(1/T) + \log P = S/T + \log P$ where ΔH_a is the heat of activation in cal./mole, R is the gas constant in cal./degree, T is the absolute temperature, and S is the slope of the Arrhenius plot. [b] As previously determined (3).

where ϵ is the equivalent absorptivity at the subscripted wavelength in mμ for aspirin anhydride (AA) or for aspirin (ASP) and where A′ is the corrected absorbance at the subscripted wavelength. The true absorbance, A, at the subscripted wavelength was corrected for the interfering absorption of the highly absorbing salicylic acid (SAL) to A′ by

$$A'_{292.5} = A_{292.5} - \epsilon^{SAL}_{292.5}[Sal] \quad \text{(Eq. 2)}$$

$$A'_{277.5} = A_{277.5} - \epsilon^{SAL}_{277.5}[Sal] \quad \text{(Eq. 3)}$$

where the salicylic acid concentration [Sal] was obtained from the absorbance at 320 mμ

$$[Sal] = A_{320}/\epsilon^{SAL}_{320} \quad \text{(Eq. 4)}$$

The logarithmic values of the calculated aspirin anhydride concentrations were plotted against time according to the first-order expression

$$\log [AA] = -kt/2.303 + \text{constant} \quad \text{(Eq. 5)}$$

and the derived first-order rate constants are given in Table I. Such plots are given in Fig. 2.

The other anhydrides studied hydrolyzed at such rates so that the spectral appearances of derived salicylic acids were no problem. The logarithm of the difference between the absorbances at any time, A, and the asymptotic value with time, A_∞, were plotted according to

$$\log [A - A_\infty] = -\frac{kt}{2.303} + \text{constant} \quad \text{(Eq. 6)}$$

and the derived first-order rate constants are also given in Table I. Such plots are given in Figs. 3 and 4.

The Arrhenius plots for the hydrolyses of these anhydrides in 75% dioxane are given in Fig. 5 and the thermodynamic quantities are given in Table I. The relation used is

$$\log k = -(\Delta H_a/2.303\text{R})(1/T) + \log P = -S/T \log P \quad (7)$$

where ΔH_a is the heat of activation in cal./mole, R is the gas constant in cal./degree, T is the absolute temperature, and S is the slope of the Arrhenius plot.

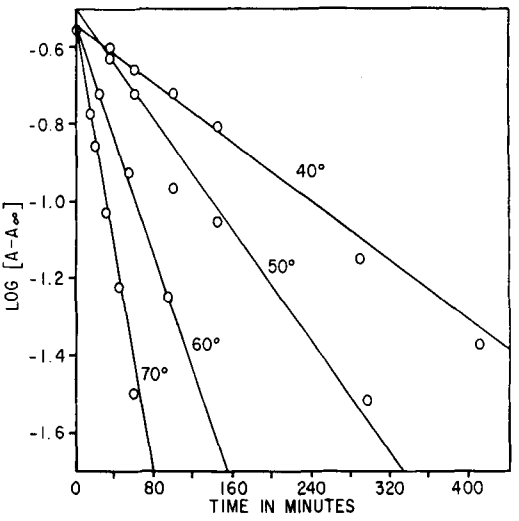


Fig. 3.—Apparent first-order plots of the loss of absorbance at the 283 mμ maximum of the mixed anhydride of aspirin and acetic acid in 75% dioxane-25% water. The A_∞ at 283 mμ (i.e., the absorbance for aspirin) for the 6.27×10^{-4} M solutions was 0.700.

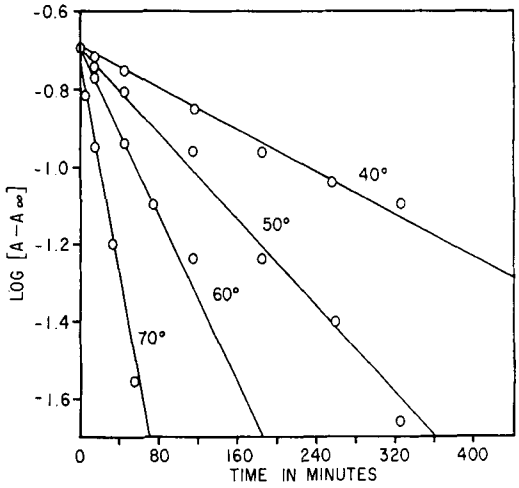


Fig. 4.—Apparent first-order plots of the loss of absorbance at the 282 mμ maximum of the mixed anhydride of acetylsalicoylsalicylic and acetic acids in 75% dioxane-25% water. The A_∞ at 282 mμ for the 3.83×10^{-4} M solutions was 0.870.

DISCUSSION

Comparison of Neutral Solvolyses of Anhydrides of *o*-Acetoxybenzoic Acid with *m*- and *p*-Substituted Benzoic Anhydrides.—In general, the neutral sol-

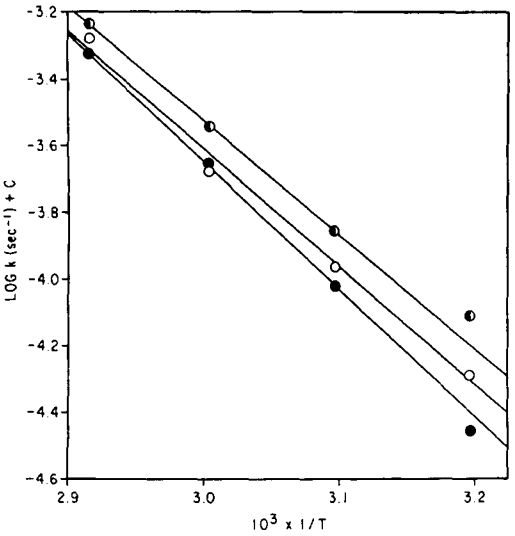


Fig. 5.—Arrhenius' plots for the hydrolysis of anhydrides in 75% dioxane-25% water. ⊙ Mixed anhydride, acetic and acetylsalicoylsalicylic acids, C = 0; ◐ mixed anhydride, acetic acid and aspirin, C = 0; ● aspirin anhydride, C = 1.

volyses of substituted benzoic anhydrides should be favored by electron withdrawing groups since nucleophilic attack is rate determining (2–6).

The acetoxy group in aspirin anhydride should possess less electron withdrawing properties than a methyl substituent (7). The *ortho* position had little effect on the heat of activation for the neutral hydrolysis of aspirin anhydride in 75% dioxane in comparison to the other *m*- and *p*-substituted benzoic anhydrides (2). The ΔH_a of 17.8 Kcal. is the same as that given for the neutral hydrolysis in 75% dioxane of the *m*- and *p*-alkyl benzoic anhydrides (2).

The unsymmetrical mixed anhydride of aspirin and acetic acid, II, and of acetylsalicoylsalicylic and acetic acids, III, may have slightly less heat of activation for their hydrolyses (see Table I).

Although this difference is not of a high order of significance, it may imply that the attack of the water molecule on the carbonyl carbon, which attack requires most of the activation energy, is slightly more facile in the case of the mixed anhydrides.

The most dramatic difference introduced by the *ortho* substituent, as expected by virtue of the proximity effect (8), is in the frequency factor. The log P value for aspirin anhydride hydrolysis is relatively quite high (see Table I) compared to all the *m*- and *p*-substituted benzoic anhydride hydrolyses listed by Berliner and Altschul (2).

The high frequency factor, or the less change in the entropy of activation, may be explained by the restricted rotation in aspirin anhydride due to the bulky *ortho* groups. On activation, the activated state has less degrees of freedom to lose compared to most *m*- and *p*-substituted benzoic anhydrides.

The lower frequency factors in the hydrolysis of the mixed anhydrides account for their higher reactivity over the symmetrical aspirin anhydride (see Table I). This difference from aspirin anhydride may be attributed to the loss in greater degrees of freedom on the preferential attack of the nucleophile on the acetyl carbonyl carbon rather than on the *o*-acetoxyphenylcarbonyl carbon. It may be expected that nucleophilic attack should take place at the acyl group of higher reactivity (4). Thus, since ethyl acetate has a greater alkaline hydrolysis rate under comparable conditions than ethyl benzoate (9), nucleophilic attack on the acetyl carbonyl carbon may be predicted.

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Coulometric Titration of Various Organomercurials and Mercury Containing Compounds

By F. HENRY MERKLE and CLARENCE A. DISCHER

The feasibility of using constant current coulometry for the analysis of various pharmaceutical compounds containing mercury has been demonstrated. Electrolytically generated thioglycollic acid served as the titrant. No preliminary treatment of the mercury compound was necessary except for those substances in which the mercury is completely bound to carbon. These latter compounds were treated with concentrated hydrochloric acid to cleave the carbon to mercury bond. The mercury content was determined with samples in the milligram range with an indicated deviation of less than one per cent from the theoretical values.

COULOMETRIC titrations are rapidly taking an important place among the instrumental techniques. Applications and advantages of this technique have been recently reviewed (1). Coulometric methods are dependent on the stoichiometry existing between the quantity of electricity and the quantity of electroactive substance transformed at an electrode during the electrolysis. The amount of material that has reacted is computed using Faraday's law.

The internal generation of a reagent by constant current coulometry has made available many unstable but potentially useful reagents for chemical analysis. One of these, organically bound sulfhydryl, has been used by Miller and Hume for the quantitative determination of different metal ions; namely, mercury (II), copper (II), gold (III), and hexacyanoferrate (III) (2, 3). It was thought that their basic technique of generating the sulfhydryl group electrochemically should be of value in the determination of mercury, organically bound, in compounds of pharmaceutical interest.

The method of Miller and Hume employs mercury (II) thioglycollate, a soluble sulfhydryl compound, as the reagent precursor for the coulometric generation of free sulfhydryl. The mercury (II) thioglycollate-thioglycollic acid couple was found to be coulometrically suitable in terms of current efficiency and potentiometric reversibility, enabling efficient titration to proceed with accurate end point detection. The reaction which supplies the reagent sulfhydryl, occurs at the mercury-pool cathode. It may be expressed by the following equation

$$Hg(RS)_2 + 2H^+ + 2e^- = Hg^0 + 2RSH \quad \text{(Eq. 1)}$$

Received January 4, 1961, from Rutgers, The State University, College of Pharmacy, Newark 4, N. J.
Accepted for publication April 7, 1961.
Abstracted from a thesis submitted to the Graduate School of Rutgers, The State University, by F. Henry Merkle in partial fulfillment of the requirements for the Master of Science degree.

and is completely reversible. This indicates that one sulfhydryl group per electron is made available by the deposition of mercury. Any free sulfhydryl may be reacted and, thus, effectively removed by reversing the current and generating mercury (II) from the mercury-pool anode according to the following equation (4)

$$Hg^0 = Hg^{++} + 2e^- \quad \text{(Eq. 2)}$$

The reactions that occur in the bulk of the solution may be described by the following equations

$$RSH + R'Hg^+ = R'HgSR + H^+ \quad \text{(Eq. 3)}$$

$$2RSH + Hg^{++} = Hg(RS)_2 + 2H^+ \quad \text{(Eq. 4)}$$

where $R'Hg^+$ represents the monoalkyl or monoaryl mercury (II) ion arising from the dissociation of the organomercurial in the untreated sample (e.g., chlormerodrin in aqueous solution). The mercury (II) in reaction (4) represents the mercury set free from organic combination by treating the sample with a strong mineral acid.

EXPERIMENTAL

Apparatus.—The constant current source described by W. N. Carson, Jr., was used (5). The circuit was so designed that a single switch synchronously controlled the flow of generating current through the cell and the timer. The electric timer[1] used is calibrated to one-tenth of a second. Potentials were measured with a pH meter[2] with a saturated calomel reference electrode and an amalgamated silver wire indicator electrode. The titration cell was a 50-ml. Pyrex beaker, to the bottom of which a side arm (glass tubing, O.D. 0.75 cm.) was fused. A platinum wire served as the electrical contact to the mercury pool through this side arm. A platinum wire helix served as the nonworking electrode. It was separated from the working solution by an agar plug containing sodium nitrate. A block diagram of the titration assembly is shown in Fig. 1.

Reagents and Solutions.—All chemicals used were reagent grade unless otherwise specified. Thiogly-

[1] A Precision Time-it, Precision Scientific Co., Chicago, Ill.
[2] Beckman model H-2.

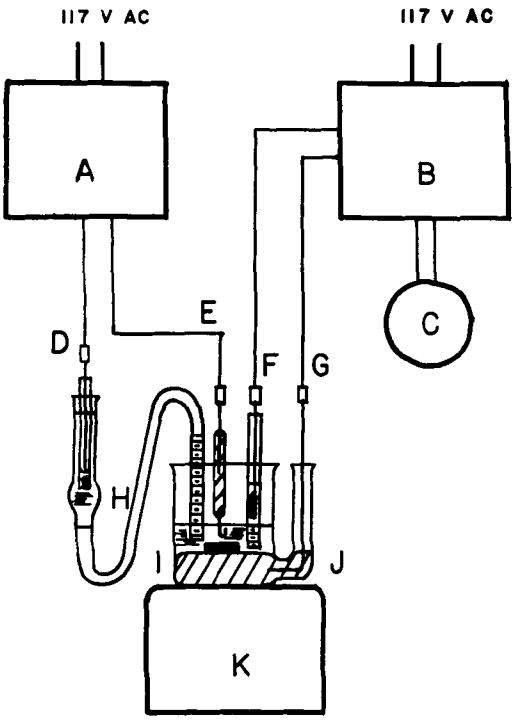


Fig. 1.—A block diagram of the titration apparatus. *A*, Beckman model H-2 pH meter; *B*, constant current source and integral milliameter; *C*, synchronous timer; *D*, saturated calomel reference electrode; *E*, mercury indicating electrode; *F*, platinum wire helix generating electrode; *G*, platinum wire contact to mercury pool electrode; *H*, saturated potassium chloride salt bridge; *I*, generating solution 5 to 7 ml.; *J*, side arm for mercury pool contact; *K*, magnetic stirrer.

collic acid (Eastman Kodak Co.), 70% practical grade and 80% analytical grade; phenylmercuric nitrate, phenylmercuric acetate, powder (The Matheson Co.); meralluride acid, powder;[3] meralluride, injectable solution; chlormerodrin, powder;[4] mercaptomerin sodium, crystals for injection[5] and injectable solution; mercury (II) salicylate powder U.S.P.; mercury (II) oxide (red) powder N.F.; ammoniated mercury powder U.S.P.

A solution of mercury (II) acetate, 0.1 *M* in acetic acid, was standardized against a standard disodium EDTA solution. The buffer used in this work (approximate pH = 5) was a solution 1.0 *M* in acetic acid and also 1.0 *M* in sodium acetate. The mercury (II) thioglycollate was prepared by mixing stoichiometric quantities of thioglycollic acid and mercury (II) chloride. The resulting precipitate was filtered and washed, and then treated with 6.0 *N* sodium hydroxide, dropwise, until completely dissolved (approximate pH = 5). This stock solution was then diluted with distilled water to 0.1 *M* with respect to mercury (II) thioglycollate.

Equal volumes of the buffer solution and the stock mercury (II) thioglycollate solution were mixed to produce the generating solution.

[3, 4] The authors wish to thank Lakeside Laboratories, Milwaukee, Wis., for the donation of these materials.

[5] The authors wish to thank Wyeth Laboratories, Philadelphia, Pa., for the donation of this material.

General Procedure.—The titration was carried out by placing about 5 to 7 ml. of the previously prepared supporting electrolyte-generating solution into the titration cell. Nitrogen was bubbled through this solution for about 5 minutes to remove any dissolved oxygen. A slow flow of gas was continued during the entire titration procedure in order to maintain an inert atmosphere. The point of maximum potential inflection was determined by generating an excess of mercury (II), then reversing the current and back titrating with sulfhydryl (Eqs. 2 and 1, respectively). Before the addition of the sample, the solution was pretitrated to the potentiometric inflection point to remove any slight excess of mercury (II) or sulfhydryl that might be present. The unknown sample was then introduced into the cell and the generating current maintained using appropriate time intervals until the original inflection point was passed (Eq. 1). After each generation period, the indicator potentials were read after equilibrium was reached. Equilibrium was attained quite rapidly, all cases requiring less than 1 minute. The end point time was then calculated from the point of maximum potential inflection. In all the titrations that were performed, the end point was necessarily overrun; and before a new sample was added, the excess sulfhydryl generated was back titrated to the potentiometric inflection point by reversing the current (Eq. 2).

Successive titrations are possible with a given portion of the supporting electrolyte-generating solution. However, the volume increase by the addition of the samples into the titration cell was found to limit the number of successive titrations that could be conducted. By maintaining a small volume in the cell, a sharper inflection point was obtained. In the case of the treated materials, the acidity of the samples added was also found to be a limiting factor. The titration curve shown in Fig. 2 is typical of all the titrations that were performed with the treated and untreated samples.

Samples Titratable without Preliminary Treatment.—The following solutions were prepared by dissolving the compounds in distilled water: phenylmercuric nitrate 0.5 Gm./L., phenylmercuric acetate 1.0 Gm./L., chlormerodrin 0.2 Gm./100 ml. A suitable sample of each solution, 1 to 2 ml., was then taken, pipetted directly into the cell, and titrated according to the procedure outlined.

Preliminary Treatment of the Samples.—The treatment of the various mercury-containing compounds varied only slightly depending on their nature. In preparing the mercury compounds, concentrated hydrochloric acid was used to cleave the mercury-carbon bond, thus releasing the mercury so that it could react with the generated sulfhydryl. The reaction that occurred with the acid for most of the alkyl and aryl salts investigated, of the type RHgOH, RHgX, and R_2Hg, is expressed by the following reaction (6)

$$R_2Hg \xrightarrow{HCl} RH + RHgCl \xrightarrow{HCl} RH + HgCl_2 \quad \text{(Eq. 5)}$$

Previous workers have accomplished similar bond cleavage by employing 50% sulfuric acid and then titrating with potassium thiocyanate (7). However,

TABLE I.—TITRATION DATA FOR CHLORMERODRIN, UNTREATED SOLUTION

No.	Current, ma.	Time, sec.	Taken, mg. Hg	Calcd. mg. Hg	Found, mg. Hg	Error, mg. × 10^{-3}
1	7.0	75.15	1.0926	0.5463	0.5469	+0.6
2	7.0	74.80	1.0926	0.5463	0.5443	−2.0
3	7.0	75.20	1.0926	0.5463	0.5472	+0.9
4	7.0	75.00	1.0926	0.5463	0.5457	−0.6
5	7.0	75.25	1.0926	0.5463	0.5476	+1.3
6	7.0	75.00	1.0926	0.5463	0.5457	−0.6
7	7.0	75.05	1.0926	0.5463	0.5461	−0.2
8	7.0	75.15	1.0926	0.5463	0.5469	+0.6

TABLE II.—TITRATION DATA FOR CHLORMERODRIN, TREATED SOLUTION

No.	Current, ma.	Time, sec.	Taken, mg. Hg	Calcd. mg. Hg	Found, mg. Hg	Error, mg. × 10^{-3}
1	7.0	149.50	1.0926	1.0926	1.0878	−4.8
2	7.0	149.60	1.0926	1.0926	1.0886	−4.0
3	7.0	150.45	1.0926	1.0926	1.0947	+2.1
4	7.0	150.50	1.0926	1.0926	1.0951	+2.5
5	7.0	149.50	1.0926	1.0926	1.0878	−4.8
6	7.0	149.80	1.0926	1.0926	1.0900	−2.6
7	7.0	150.30	1.0926	1.0926	1.0937	+1.1
8	7.0	150.35	1.0926	1.0926	1.0942	+1.6
9	7.0	150.10	1.0926	1.0926	1.0922	−0.4
10	7.0	149.75	1.0926	1.0926	1.0897	−2.9

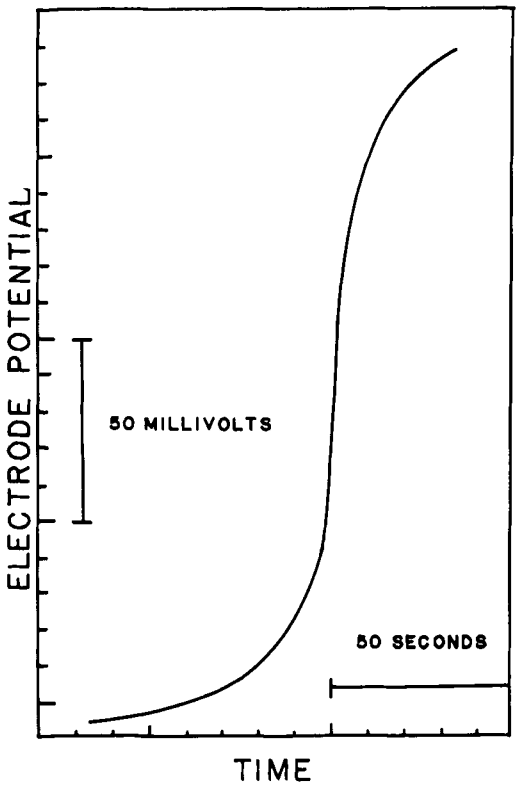


Fig. 2.—Typical titration curve obtained for chlormerodrin treated sample.

this earlier method is not applicable when hydrochloric acid or chloride ions are present.

With the following substances, it was necessary to vary the procedure as follows: 50 ml. of phenylmercuric nitrate solution (0.05%) and phenylmercuric acetate solution (0.10%) were pipetted into separate 100-ml. volumetric flasks. The solutions were boiled down to approximately 25 ml., and 5 ml. of concentrated hydrochloric acid was added, dropwise. After carefully boiling until complete solution was accomplished (usually 10 to 15 minutes), the solutions were neutralized with 6 *N* sodium hydroxide to a pH of approximately 5. Upon cooling, the solutions were diluted to the mark with distilled water.

Any loss of mercury (II) chloride, due to volatilization during the boiling procedure, was found to be negligible.

The two injectable solutions were sampled with a micrometer syringe.[6] Predetermined volumes (0.5 or 1.0 ml.) of these solutions were introduced into 50-ml. volumetric flasks containing 1 to 2 ml. of the concentrated acid. Distilled water, 10–15 ml., was added to the solutions before they were gently boiled. After solution was accomplished, they were cooled to room temperature and diluted to the mark with distilled water.

When solid materials were to be analyzed, appropriate samples (100 or 200 mg.) of the dry materials were weighed and then washed into a 100-ml. volumetric flask containing 2 ml. of the concentrated acid with 15 ml. of distilled water. In the case of mercury (II) salicylate, more distilled water was added and the heating time extended to dissolve the material completely. After cooling the solutions, they were diluted to the mark with distilled water. In the case of the samples of the injectable solutions and the dry materials, the neutralization step was found unnecessary since a minimum of acid was used.

Titration of Treated Samples.—The treated samples (1 to 2 ml.) were taken from the solutions, prepared as previously described, and pipetted directly into the titration cell.

[6] "Agla" brand micrometer syringe, Wellcome Research Laboratories, Beckenham, Kent, England (0.5-ml. capacity, with the precision of delivering 0.01 ml. to ±0.5%).

TABLE III.—SUMMARY OF ALL DATA OBTAINED IN TITRATING MERCURY COMPOUNDS

Sample Taken	No. Determinations Made	Sample, mg. Hg	Thoretical e.p., mg. Hg[b]	Found, Av. mg. Hg	Mean Dev.[c]	Std. Dev.[d]
Phenylmercuric acetate[a]	8	1.1914	0.5957	0.5933	0.0023	0.0035
Phenylmercuric acetate	9	0.5957	0.5957	0.5954	0.0034	0.0041
Phenylmercuric nitrate[a]	10	0.6100	0.3050	0.3055	0.0030	0.0041
Phenylmercuric nitrate	10	0.3050	0.3050	0.3050	0.0022	0.0011
Mercuhydrin acid	9	0.6378	0.6378	0.6407	0.0029	0.0036
Mercuhydrin injection	8	0.3900	0.3900	0.3885	0.0011	0.0012
Mercaptomerin sodium	15	1.3234	0.6617	0.6613	0.0011	0.0015
Mercaptomerin sodium injection	8	0.8270	0.4135	0.4154	0.0017	0.0022
Ammoniated mercury	10	0.7958	0.7958	0.7969	0.0020	0.0026
Mercury salicylate	8	0.5958	0.5958	0.5987	0.0020	0.0024
Mercuric oxide (red)	10	0.7408	0.7408	0.7406	0.0012	0.0015
Chlormerodrin[a]	8	1.0926	0.5463	0.5463	0.0009	0.0011
Chlormerodrin	10	1.0926	1.0926	1.0914	0.0026	0.0029

[a] Samples untreated; all others are treated as described.
[b] Calculated on the basis of available mercury to react with the sulfhydryl.

[c] $d = \frac{\Sigma|X_i - \bar{X}|}{n}$ [d] $s = \sqrt{\frac{\Sigma(X_i - \bar{X})^2}{(n-1)}}$

RESULTS AND DISCUSSION

Titration Data.—Tables I and II indicate typical data obtained in the titration of chlormerodin, untreated and treated samples, respectively. Table III is a summary of all the data obtained in titrating the various organomercurials.

Conclusions.—The experimental data indicated that electrolytically generated sulfhydryl can serve as a titrant in the determination of mercury (II) released from organic combination. In the samples treated with concentrated hydrochloric acid, the total mercury can be determined with the exception of mercaptomerin sodium. In the latter case, only half the total mercury content can be titrated. This indicates that the hydrochloric acid treatment is effective in cleaving the mercury-carbon bond, rendering the mercury available for reaction with the generated sulfhydryl, while insufficient to disrupt the mercury-sulfur bond.

A significant advantage to the general application of this method is the possibility of titrating the material directly without any preliminary treatment. The direct titration of the untreated solutions of chlormerodrin, phenylmercuric acetate, and phenylmercuric nitrate showed that this was possible. In these determinations, one-half the total mercury was titratable.

The chief difficulty with methods now generally available, involving direct titration with thiocyanate is the presence of chlorides. This drawback is overcome with the present procedure. In addition, the drastic heating usually necessary to decompose organic mercurials is eliminated since the hydrochloric acid treatment is effective in the carbon-mercury bond cleavage.

Although interferences by such ions as copper (II), gold (III), and hexacyanoferrate (III) are possible, their presence in the compounds studied is virtually nonexistent and, therefore, introduce no difficulty in the analyses.

The precision of the titrations is as good as, or better than, that with the methods ordinarily used for other mercury compounds.

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Biosynthesis of Hyoscyamine and Scopolamine in *Datura stramonium*

By E. W. UNDERHILL† and H. W. YOUNGKEN, Jr.

Datura stramonium L. plants were allowed to metabolize certain C^{14}-labeled compounds for 10 days following which hyoscyamine and scopolamine were isolated and purified. Of the compounds employed, phenylalanine-3-C^{14}, phenylacetic acid-1-C^{14}, and sodium acetate-2-C^{14} were the most efficient precursors, followed by sodium propionate-2-C^{14}, sodium formate-C^{14}, and serine-3-C^{14} in that order; zinc lactate-2-C^{14} proved to be a comparatively inefficient precursor. The distribution of C^{14} within the alkaloids was entirely in their tropic acid moiety when phenylalanine and phenylacetic acid were used as precursors, and almost entirely in their tropine or scopine portions when acetate, propionate, formate, and serine were employed. The samples of tropic acid obtained, following administration of phenylalanine and phenylacetic acid, were degraded and the location of the C^{14} determined.

A NUMBER of theories on the biosynthesis of of the tropane moiety of hyoscyamine and scopolamine have been proposed and these have recently been reviewed (1). Experiments involving C^{14}-labeled compounds as precursors of this portion of the alkaloids have provided positive results in the case of ornithine-2-C^{14} (2), methionine-C^{14} (3), and acetate-C^{14} (4, 5), while negative results have been obtained when putrescine-1,4-C^{14} (6) and citric acid-3-C^{14} (7) were employed. Recently attention has been focused upon the formation of the tropic acid moiety of these alkaloids. Trautner (8) has suggested that tropic acid, itself possessing an isopropylbenzene skeleton, may be derived from the the terpenes, limonene and terpinene. Shortly after this work was commenced, Wenkert (9) suggested that this aromatic branched-chain acid may be derived from an aldol product of formaldehyde and prephenic acid. A number of routes leading from such an aldol product to tropic acid are possible and these have been elaborated upon in the experimental work reported by Leete (10), who administered DL-phenylalanine-3-C^{14}, sodium formate-C^{14}, and formaldehyde-C^{14} to *Datura stramonium*. Of the compounds employed by Leete, only phenylalanine was a precursor of tropic acid and in this instance the activity resided in the carbon adjacent to the aromatic group. Goodeve and Ramstad (11) have found that the administration of tryptophan-3-C^{14} to *D. stramonium* yielded hyoscyamine with the totality of its activity in the carboxyl carbon of tropic acid.

In the present paper experiments are reported in which the validity of the following three hypotheses for tropic acid biosynthesis was determined: (*a*) Acetate, an effective precursor of isoprenoid compounds might also be expected to become incorporated into tropic acid via the terpene pathway suggested by Trautner (8). (*b*) The aromatic branched-chain structure of tropic acid may be formed through the condensation of shikimic acid with the alpha carbon of a 3-carbon acid such as lactate or propionate. (*c*) Tropic acid may be formed by the attack of such an "active" 1-carbon compound as formate or the beta carbon from serine at the alpha carbon of phenylacetic acid or its coenzyme derivative (derived by oxidative decarboxylation of the transaminated product of phenylalanine, *viz.*, phenylpyruvic acid).

EXPERIMENTAL

C^{14}-Sample Preparation and Counting.—Radioactive samples were assayed by combustion of the material to carbon dioxide and converting the evolved gas to barium carbonate. The Van Slyke-Folch wet combustion mixture, prepared according to Calvin, *et al.* (12), was employed in an apparatus similar to that described by Claycomb, *et al.* (13). Barium carbonate samples were counted in a windowless flow counter at a finite thickness and the activity corrected to infinite thinness.

Cultivation of Plants.—*Datura stramonium* plants were grown in soil until the most mature plants bore flower buds (44 days). The roots of 24 plants of uniform size and appearance (no flower buds appearing) were washed free of soil and the plants placed in hydroponic culture. After the plants had remained in the hydroponic culture for 1 week, the roots of each plant were removed to within approximately $^1/_8$ inch of the crown. The purpose of the root excision was to provide a soil-free root system wherein a high rate of alkaloid synthesis might be expected to occur in the abundant quantity of young, actively metabolizing root tissue which formed during the following 10 days.

Administration of C^{14}-Labeled Compounds to Plants.—At the end of the 10-day root regeneration

Received April 7, 1961, from the College of Pharmacy, University of Rhode Island, Kingston.
Accepted for publication June 6, 1961.
Based in part, on a Ph.D. dissertation submitted by E. W. Underhill to The Graduate School, University of Rhode Island, June 1960.
This study was supported in part by United States Public Health Service grant MY3319 and in part by a grant from the Smith, Kline and French Foundation.
† Recipient of a National Research Council of Canada Special Scholarship and Edwin Leigh Newcomb Award in Pharmacognosy for 1961.

period, the C^{14}-labeled compounds were administered to the plants. Eight groups of three plants each were employed as follows: one group was selected as a control and as such received no radioactive compounds; the remaining seven groups were separately fed the following radioactive compounds, purchased from commercial sources: *dl*-phenylalanine-3-C^{14}, phenylacetic acid-1-C^{14}, sodium acetate-2-C^{14}, sodium propionate-2-C^{14}, sodium formate-C^{14}, *dl*-serine-2-C^{14}, and *dl*-zinc lactate-2-C^{14}.

The plants were individually placed in small beakers containing equal quantities (0.07 m*M* per plant) of the C^{14}-labeled compounds dissolved in 2 to 3 ml. of water. The illuminated plants absorbed their respective solutions within 20 minutes; the last traces were washed into the plant by addition of at least 12 successive 2-ml. portions of water to the beaker as it approached dryness. The plants were then transferred to hydroponic culture and were allowed to metabolize in a greenhouse under ordinary conditions of sunlight for a further period of 10 days.

Isolation and Purification of Alkaloids.—The plant material was dried at 65° for 36 hours and then ground in a semimicro Wiley mill to a No. 40 powder. The powdered material was extracted with ether in a Soxhlet apparatus according to the method described for this plant in the N.F. X (14). Further purification of the combined alkaloids was effected by extraction of the bases from the ethereal solution into acidulated water and then into chloroform. Hyoscyamine was separated from scopolamine by countercurrent liquid-liquid extraction using a phosphate buffer (0.2 *M*, pH 6.8) and chloroform as the two phases (15). Final purification of the individual alkaloids was carried out on Celite[1] columns after the method of Evans and Partridge (16). The amount of column-purified alkaloid was determined by colorimetric assay (17).

Hydrolysis of Alkaloids.—Those alkaloids possessing sufficient radioactivity to warrant further study were hydrolyzed to tropine or scopine and tropic acid in order to determine the distribution of C^{14} between these two portions of the alkaloids.

Sufficient nonradioactive alkaloid was added to the column-purified alkaloids to provide a sample of known weight between 100 and 150 mg. Complete hydrolysis was accomplished by dissolving the alkaloid in 35 ml. of water to which was added 10 ml. of 2 *N* sodium hydroxide and heating the mixture on a water bath for 2 hours. Tropine or scopine was extracted from the hydrolysate with five 25-ml. portions of chloroform. Following acidification of the hydrolysate, tropic acid was recovered by extraction with several portions of a mixture of isopropanol (1 part) and chloroform (3 parts).

Evaporation of the isopropanol-chloroform mixture yielded a light brown residue from which tropic acid was recovered by sublimation at 114–115° and 1–2 mm. Hg pressure. Crystallization of the sublimed acid gave pure tropic acid, m.p. 117–118°; yield based on the alkaloid was 80% or more.

Tropine was purified by formation of its picrate derivative and recrystallization of tropine picrate from water, m.p. 290–293°; yield 30 to 40% based on hyoscyamine. Repeated attempts to form the picrate of scopine failed to provide a derivative with a sharp melting point.

Degradation of Tropic Acid.—Two methods of degradation were employed. The first involved permanganate oxidation of tropic acid to benzoic acid while the second involved the decarboxylation of the branched-chain acid and recovery of the liberated carbon dioxide.

Tropic acid (30 to 50 mg.) was refluxed with 250 mg. of potassium permanganate and 50 mg. of sodium hydroxide in 5 ml. of water for 2 hours. The hot solution was filtered, acidified, and then extracted with successive 20-ml. portions of ether. Removal of the solvent left a light brown residue which, on sublimation, yielded 10 to 15 mg. of long, colorless crystals of benzoic acid, m.p. 122°.

Decarboxylation of tropic acid was accomplished by refluxing 10 to 20 mg. of the acid with 10 mg. of cupric oxide in 3 ml. of quinoline for 1 hour. The reaction was carried out in an atmosphere of nitrogen and the evolved carbon dioxide was collected in carbon dioxide-free sodium hydroxide. The yield of carbonate ranged from 95 to 110% of the theoretical after allowance was made for the carbonate which formed when appropriate blanks were employed.

The styrene and/or polystyrene, which would be expected to be formed as a result of tropic acid decarboxylation, was recovered from the reaction mixture in the following manner. The quinoline solution was acidified and then extracted with seven successive 10-ml. portions of ether. The combined ether extract was washed several times, first with acidulated water and then with dilute sodium hydroxide. Evaporation of the washed ether extract left a brown residue.

RESULTS

The relative effectiveness of the administered compounds as precursors of hyoscyamine and scopolamine can be seen by comparing the dilution values given in Table I. Phenylalanine, phenylacetic acid, and sodium acetate, which gave hyoscyamine and scopolamine showing the least dilution of C^{14}, were the best alkaloid precursors of the seven compounds tested. Sodium propionate, sodium formate, and serine were less efficient precursors, while the radioactive carbon from zinc lactate was only slightly incorporated into the alkaloids. The similarity in dilution values for the two alkaloids, upon administration of any one precursor, suggests that both hyoscyamine and scopolamine were being formed at the same time within the plants.

Tables II and III show the distribution of C^{14} within the alkaloids as determined by the specific activities of the hydrolysis products, tropic acid and tropine. The specific activity for scopine could not be determined due to the questionable composition of its picrate derivative and the specific activity values listed for this base have been derived by calculation. It is apparent from the results that, of the seven compounds administered to the plants, only phenylalanine and phenylacetic acid were precursors of tropic acid specifically. The radioactivity derived from sodium propionate also occurred to a limited extent in the acid but the majority of its activity, like that of acetate, formate, and serine, was located in tropine or scopine.

[1] A product of Johns-Manville, Celite Division, New York, N. Y.

TABLE I.—COMPARISON OF C^{14}-LABELED COMPOUNDS AS PRECURSORS OF ALKALOIDS IN *Datura Stramonium*

Compound Administered	Amount Used, μM/Gm. Dried Plant	Specific Activity, c.p.m. × 10^5/mM	Specific Activity of Alkaloids, c.p.m. × 10^5/mM Hyoscyamine	Specific Activity of Alkaloids, c.p.m. × 10^5/mM Scopolamine	Dilution Values Hyoscyamine	Dilution Values Scopolamine
dl-Phenylalanine-3-C^{14}	7.33	1,380	9.73	7.30	142	189
Phenylacetic acid-1-C^{14}	9.90	292	1.24	1.43	235	204
Sodium acetate-2-C^{14}	8.70	1,160	12.22	9.68	97	120
Sodium propionate-2-C^{14}	9.23	358	0.99	0.78	361	459
Sodium formate-C^{14}	6.58	750	1.45	0.97	517	772
dl-Serine-3-C^{14}	7.32	1,525	1.30	1.08	1,173	1,413
dl-Zinc lactate-2-C^{14}	12.00	352	0.03	0.01	12,000	25,500

TABLE II.—DISTRIBUTION OF C^{14} IN HYOSCYAMINE

Compound Administered	Specific Activity of Hydrolysis Products, c.p.m./mM × 10^5 Tropic Acid	Tropine	% Activity[a] Found in Hydrolysis Products Tropic Acid	Tropine
dl-Phenylalanine-3-C^{14}	9.60	0.00	100	0
Phenylacetic acid-1-C^{14}	1.02	0.00	100	0
Sodium acetate-2-C^{14}	0.40	8.55	5	95
Sodium propionate-2-C^{14}	0.14	0.85	14	86
Sodium formate-C^{14}	0.05	1.35	4	96
dl-Serine-3-C^{14}	0.06	1.40	4	96
dl-Zinc lactate-2-C^{14}	[b]	[b]	[b]	[b]

[a] % Activity = $\frac{\text{specific activity of hydrolysis product}}{\text{sum of specific activities of both hydrolysis products}} \times 100$.

[b] Insufficient material or radioactivity to permit hydrolysis.

TABLE III.—DISTRIBUTION OF C^{14} IN SCOPOLAMINE

Compound Administered	Specific Activity of Hydrolysis Products, c.p.m./mM × 10^5 Tropic Acid	Scopine[a]	% Activity[b] Found in Hydrolysis Products Tropic Acid	Scopine[a]
dl-Phenylalanine-3-C^{14}	7.32	0.00	100	0
Phenylacetic acid-1-C^{14}	1.61	0.00	100	0
Sodium acetate-2-C^{14}	0.24	9.43	2	98
Sodium propionate-2-C^{14}	[c]	[c]	[c]	[c]
Sodium formate-C^{14}	0.06	0.91	7	93
dl-Serine-3-C^{14}	0.04	1.04	4	96
dl-Zinc lactate-2-C^{14}	[c]	[c]	[c]	[c]

[a] Calculated by difference of specific activities of alkaloid and tropic acid.

[b] % Activity = $\frac{\text{specific activity of hydrolysis product}}{\text{sum of specific activities of both hydrolysis products}} \times 100$

[c] Insufficient material or radioactivity to permit hydrolysis.

While all of the tropic acid obtained following hydrolysis of the alkaloids possessed radioactivity, only four samples were sufficiently active to be considered for degradation; these were obtained when phenylalanine and phenylacetic acid were used as precursors. The original hypothesis concerning the biosynthesis of tropic acid from phenylalanine suggested that the carbon skeleton of this amino acid would go to form the phenyl, alpha, and carboxyl carbons of tropic acid. On this premise, the activity within tropic acid should be located in the carbon adjacent to the benzene ring since phenylalanine-3-C^{14} was employed as the precursor. This hypothesis is supported by the results of permanganate oxidation of tropic acid to benzoic acid (Table IV) wherein essentially all of the activity present in the tropic acid obtained from both alkaloids was contained in the benzoic acid.

The tropic acid recovered following administration of phenylacetic acid was decarboxylated since, according to the starting hypothesis, the C^{14} from

TABLE IV.—DEGRADATION OF TROPIC ACID

Compounds Administered	Alkaloid Yielding Tropic Acid	Specific Activity, c.p.m./mM Tropic Acid	Specific Activity, c.p.m./mM Benzoic Acid
dl-Phenylalanine-3-C^{14}	Hyoscyamine	9.60 × 10^5	9.23 × 10^5
dl-Phenylalanine-3-C^{14}	Scopolamine	7.32 × 10^5	7.05 × 10^5

TABLE V.—RADIOACTIVITY PRESENT IN DECARBOXYLATED PRODUCTS OF TROPIC ACID FROM PHENYLACETIC ACID-1-C^{14}

Alkaloid Containing Tropic Acid	Radioactivity Present c.p.m. Tropic Acid Prior to Decarboxylation	Radioactivity Present c.p.m. Decarboxylated Products	% Radioactivity Present in Decarboxylated Products
Hyoscyamine	6,740	5,470	81
Scopolamine	11,060	8,400	76

phenylacetic acid-1-C^{14} should be present in the carboxyl carbon of tropic acid. However, the liberated carbon dioxide, collected and counted as barium carbonate, proved to be nonradioactive. The decarboxylation products, believed to be styrene and polystyrene, were recovered from the reaction mixture and their total activity determined. Table V shows the radioactivity recovered in these styrene products expressed as a percentage of the total radioactivity present in the tropic acid samples prior to decarboxylation. While most of the radioactivity was recovered, failure to obtain complete recovery is believed to be due to incomplete extraction of the degradation products into ether and also incomplete decarboxylation.

DISCUSSION

In view of Leete's success (10), as well as that reported here, in isolating both radioactive hyoscyamine and scopolamine, it would now appear that the failure of earlier workers (2, 3) to obtain radioactive scopolamine in the presence of C^{14}-labeled hyoscyamine was a function of the maturity of the plants in relation to the time of precursor administration. Preliminary experiments to those reported in this paper have shown that hyoscyamine contained most, if not all, of the C^{14} within the two alkaloids when these same seven radioactive compounds were administered to mature *D. stramonium* (plants bearing fruit). The failure to obtain radioactive scopolamine in the presence of C^{14}-labeled hyoscyamine using mature *D. stramonium* plants has been demonstrated by Romeike and Fodor (18).

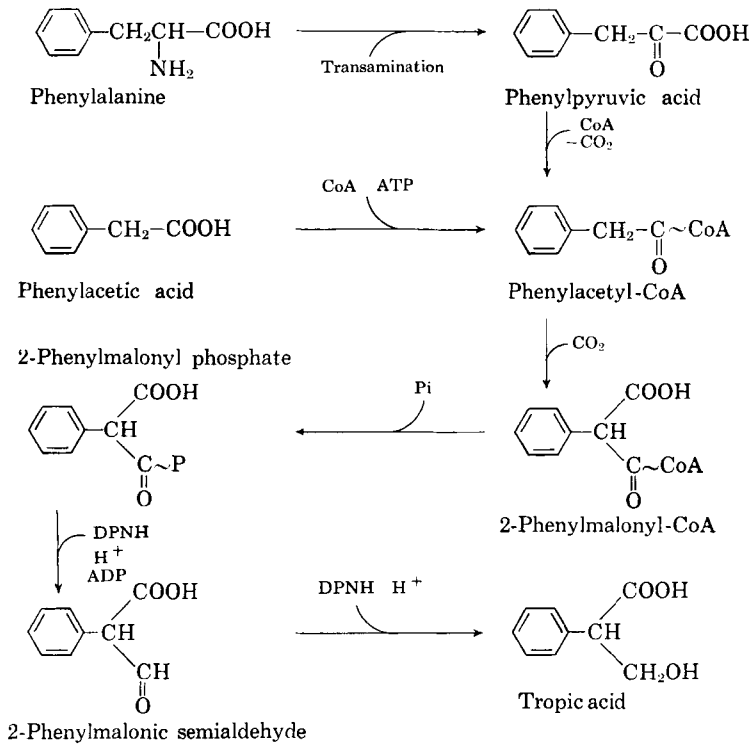


Fig. 1.—Proposed pathway for tropic acid biosynthesis.

The foregoing results provide no evidence which would support two of the proposed hypotheses for tropic acid biosynthesis, namely, the participation of acetate via the isoprene pathway suggested by Trautner (8) and the condensation of shikimic acid with lactate or propionate. The third hypothesis involved the formation of phenylacetic acid or its coenzyme derivative from phenylalanine followed by a 1-carbon attack at its alpha carbon by formate or the beta carbon of serine. This hypothesis has been substantiated in part by the results obtained in that both phenylalanine-3-C^{14} and phenylacetic acid-1-C^{14} were efficient precursors of tropic acid.

Two major discrepancies in the experimental results and the third hypothesis were met. First, the activity derived from formate-C^{14} and serine-3-C^{14} was not found in tropic acid but rather in the tropane moiety of the alkaloids. The second discrepancy, and the one which has provided the greatest interest and speculation, was that the radioactivity present in tropic acid derived from phenylacetic acid-1-C^{14} was not in the carboxyl group as was expected but is believed to have been present in the hydroxymethyl carbon.

These findings have led us to propose the following pathway for tropic acid biosynthesis (Fig. 1) which, in addition to accounting for the experimental findings, suggests the direct participation of carbon dioxide in the formation of the branched chain. The essential points in this proposal are as follows: (*a*) the addition of carbon dioxide to phenylacetyl-Co.A in a manner analogous to the formation of malonyl-Co.A from acetyl-Co.A in fat biosynthesis (19); (*b*) reduction in two stages of 2-phenylmalonyl-Co.A to tropic acid.

Degradative studies were not carried out to ascertain the location of the C^{14} which was predominantly incorporated into tropine and scopine upon administration of formate, serine, acetate, and propionate; hence, the full significance of these results is not possible to determine. It was not surprising that the C^{14} derived from formate-C^{14} and serine-3-C^{14} was incorporated into these bases since these compounds are known precursors of N-methyl groups in alkaloids. Leete (10) administered formaldehyde-C^{14} and formate-C^{14} to *D. stramonium* in addition to phenylalanine-3-C^{14}, and found that the activity from formate did in fact reside in the N-methyl of hyoscyamine. Acetate-2-C^{14} and propionate-2-C^{14} were very efficient precursors of tropine and scopine. Wenkert (9) and Birch (20), have suggested acetate as a possible precursor of the "acetone" moiety of tropine and scopine. Recently, Kaczkowski, Schütte, and Mothes (4, 5) have clearly demonstrated the participation of acetate in the formation of the tropine portion of hyoscyamine. Degradation of the alkaloid showed that the radioactivity, derived from acetate-1-C^{14}, was in carbon atom 3 while that derived from acetate-2-C^{14} was in carbon atoms 2 and 4. A further possibility of the location of C^{14} from both acetate-2-C^{14} and propionate-2-C^{14} stems from work on the biosynthesis of nicotine. Griffith and Byerrum (21) found that, following administration of these similarly labeled compounds to *Nicotiana rustica*, half of the C^{14} within nicotine was located in the pyrrolidine ring.

Work is continuing to provide more definitive information concerning the participation of propionate and carbon dioxide in the biosynthesis of hyoscyamine and scopolamine.

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Influence of Nonequilibrium Sample Temperature on Stability Predictions Extrapolated from Elevated Temperature Studies

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Assay results for a single sample stored in an elevated temperature oven for a known period of time may be in error if instantaneous heating and cooling of the sample is not achieved. For usual kinetic studies however, the storage behavior of a group of samples which have been placed in the oven at the same time is followed. Theoretical and experimental considerations are presented to show that in the case of apparent first-order reactions the assay values for all samples in the group are subjected to the same degree of error. Rates of degradation calculated from the assay data are identical to those measured under isothermal conditions. Consequently, decomposition rates obtained under the usual oven storage conditions, where time lags in heating and cooling samples exist, give useful information for estimating product stability.

CONSIDERABLE PROGRESS has been made in recent years in applying chemical kinetic principles for the prediction of the shelf life of pharmaceutical products. The techniques employed are gaining general acceptance in the pharmaceutical industry and have been the subject of an increasing number of literature reports.

In one early investigation, Garrett and Carper (1) predicted the color stability of a multisulfonamide preparation using colorimetric measurements of samples subjected to thermally accelerated degradation. Later, Swintosky, *et al.* (2), used chemical kinetics in predicting the shelf life of oral penicillin G procaine suspensions. In another report, Garrett (3) examined the thermal degradation of ascorbic acid, vitamin B_{12}, folic acid, and other components of a multivitamin preparation. The author was able to arrive at satisfactory estimates of room temperature stability from data obtained under accelerated test conditions. McLeod (4) used a similar approach to predict expiration dates for other multivitamin preparations.

These reports and others on the subject (5–7) have suggested a simple experimental procedure for the evaluation of product stability. The procedure generally followed involves storing the product, preferably packaged in its final market container, in three or four individual ovens maintained at constant elevated temperature. Periodically samples are removed from these ovens and assayed. The loss of active ingredient is calculated, and rates of decomposition for the

Received April 28, 1961, from the Warner-Lambert Research Institute, Morris Plains, N. J. and the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
Accepted for publication June 20, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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Influence of Nonequilibrium Sample Temperature on Stability Predictions Extrapolated from Elevated Temperature Studies

By MORTON W. SCOTT† and LEON LACHMAN‡

Assay results for a single sample stored in an elevated temperature oven for a known period of time may be in error if instantaneous heating and cooling of the sample is not achieved. For usual kinetic studies however, the storage behavior of a group of samples which have been placed in the oven at the same time is followed. Theoretical and experimental considerations are presented to show that in the case of apparent first-order reactions the assay values for all samples in the group are subjected to the same degree of error. Rates of degradation calculated from the assay data are identical to those measured under isothermal conditions. Consequently, decomposition rates obtained under the usual oven storage conditions, where time lags in heating and cooling samples exist, give useful information for estimating product stability.

CONSIDERABLE PROGRESS has been made in recent years in applying chemical kinetic principles for the prediction of the shelf life of pharmaceutical products. The techniques employed are gaining general acceptance in the pharmaceutical industry and have been the subject of an increasing number of literature reports.

In one early investigation, Garrett and Carper (1) predicted the color stability of a multisulfonamide preparation using colorimetric measurements of samples subjected to thermally accelerated degradation. Later, Swintosky, *et al.* (2), used chemical kinetics in predicting the shelf life of oral penicillin G procaine suspensions. In another report, Garrett (3) examined the thermal degradation of ascorbic acid, vitamin B_{12}, folic acid, and other components of a multivitamin preparation. The author was able to arrive at satisfactory estimates of room temperature stability from data obtained under accelerated test conditions. McLeod (4) used a similar approach to predict expiration dates for other multivitamin preparations.

These reports and others on the subject (5–7) have suggested a simple experimental procedure for the evaluation of product stability. The procedure generally followed involves storing the product, preferably packaged in its final market container, in three or four individual ovens maintained at constant elevated temperature. Periodically samples are removed from these ovens and assayed. The loss of active ingredient is calculated, and rates of decomposition for the

Received April 28, 1961, from the Warner-Lambert Research Institute, Morris Plains, N. J. and the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
Accepted for publication June 20, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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several temperatures are obtained. These rates are plotted according to the Arrhenius relationship. Extrapolation of the Arrhenius plot gives the estimate of room temperature stability.

Errors which may arise in this procedure were recently discussed (8). In particular, it was observed that considerable time may elapse before samples placed in elevated temperature ovens reach thermal equilibrium. A time lag also may occur while the sample returns to room temperature after it is removed from the oven. These time lags lead to incorrect estimates of the actual storage interval at elevated temperature to which the samples are exposed. In order to correct for these errors, the concept of "Equilibrium Temperature Time Equivalent" or ETTE has been introduced. The ETTE is defined as the effective time of storage at oven temperature. It is calculated from the assay data by computer techniques, provided that an estimate of the heat transfer coefficients can be obtained.

Because of the increasing importance of accelerated testing procedures in industry, it seemed appropriate to explore in detail the errors introduced in stability predictions by heating and cooling lag times. This was done by using a theoretical and experimental approach to the problem.

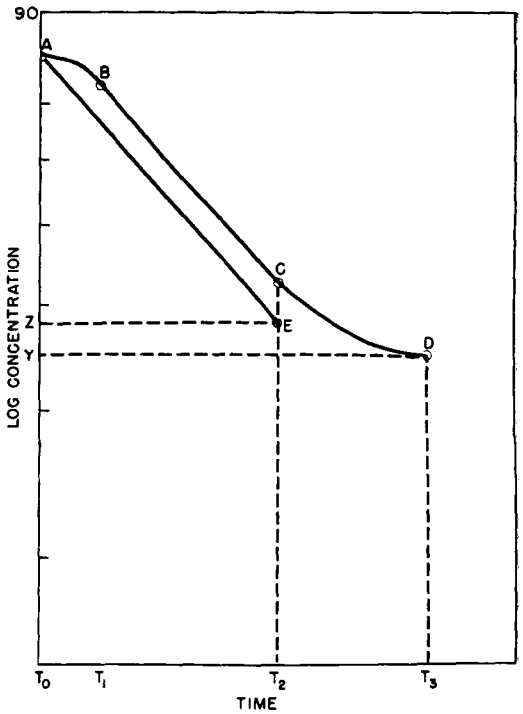


Fig. 1.—Time-concentration history for apparent first-order decomposition with heating and cooling lags, line *A-D;* and without lag, line *A-E.*

THEORETICAL TREATMENT

Figure 1 illustrates the decomposition history of a hypothetical pharmaceutical product stored at elevated temperature when degradation occurs by apparent first-order reaction. At zero time (T_0) the sample is placed in the storage oven and proceeds to warm to the equilibrium storage temperature at time T_1. During this heating interval the product temperature rises continuously, and breakdown occurs at an increasing rate. This is indicated by the changing slope of the line *A-B*. At time T_1, the sample temperature has reached its equilibrium value, and decomposition has reached its limiting rate. This maximum rate of breakdown continues at a constant level over the remaining time interval of actual storage in the oven. This period is indicated by the line *B-C*. At time T_2, the sample is removed from the oven and cooled to room temperature. While cooling, the decomposition rate gradually decreases as indicated by the changing slope of line *C-D*. The assay value obtained at the conclusion of the study, time T_3, is found to be Y units.

A hypothetical situation where the product is instantly heated to oven temperature, held at this temperature over the entire storage interval, and then instantly cooled back to room temperature is shown as a dashed line in Fig. 1. In this case, the rate of decomposition is constant and results in an assay value of Z units at time T_2. This value is higher than that obtained in the first example (Y units). The difference between Y and Z represents the error arising because of heating and cooling lag times. The presence of this $Y-Z$ error was illustrated in a similar fashion by Erickson, *et al.* (8), and served as the starting point for the development of the ETTE concept.

It is important to recognize, however, that in the usual kinetic study we are not primarily interested in the absolute assay value of any one particular sample. What is desired for the stability prediction are the rates of decomposition at the various elevated temperatures. These rates are obtained generally by following the decomposition history of a series of samples which have been placed in oven storage.

Figure 2 illustrates a theoretical decomposition history for a series of samples stored at oven temperature for various time intervals. First-order kinetic principles have been assumed to apply. It was also assumed that all samples were placed in the oven at the same time and heated at the same rate to the equilibrium oven temperature. Further, it was assumed that all samples cool to room temperature at the same rate after they are removed from the oven. These assumptions are completely in accord with industry practice and are justified by the results of Erickson and co-workers (9). It is important to note that in the present study no assumptions were made (or required) that the rates of heating (to oven temperature) or cooling (to room temperature) are equal to each other.

For the analysis shown in Fig. 2, all samples are placed in the oven at time T_0 and heated to equilibrium oven temperature at time T_1. The first sample is held in the oven until time T_2 and then cooled to room temperature by time T_3 giving an assay

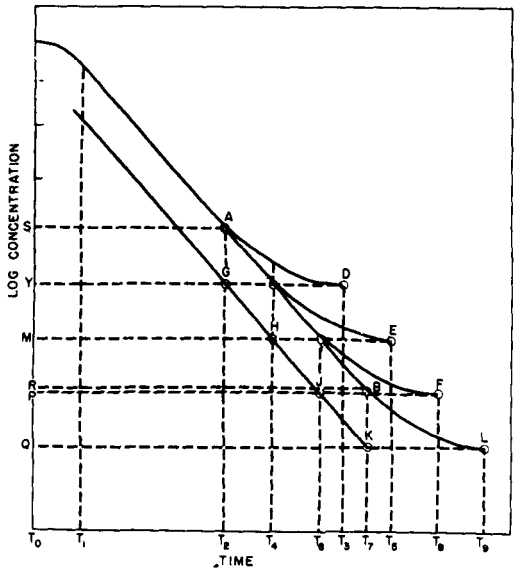


Fig. 2.—Time-concentration history for a series of samples decomposing under apparent first-order kinetics.

value of Y units. This plot is identical with that shown in Fig. 1.

The second sample is held at oven temperature until time T_4 and then removed from the oven and cooled to room temperature at time T_5, giving a final assay value of M units. Similarly, samples 3 and 4 are held at oven conditions for times T_6 and T_7, respectively, and then allowed to cool to room temperature to yield final assay values of P and Q units, respectively.

The individual assay values of Y, M, P, and Q units, together with the respective storage time intervals for each sample, constitute the entire data collection obtained for this particular temperature condition. It must be noted, however, that although assays are normally performed on samples which have cooled to room temperature, the storage interval is usually measured to the time the sample is actually removed from the oven. The data points used to plot the breakdown rates will therefore have the coordinates of (Y, T_2), (M, T_4), (P, T_6), and (Q, T_7) for the first, second, third, and fourth samples, respectively. These points have been designated in Fig. 2 by the letters G, H, J, and K.

A straight line has been drawn through the points G, H, J, and K in Fig. 2. Further, the slope of this constructed line has been set equal to the slope of the line A-B, which in turn equals the slope of line B-C in Fig. 1. The analysis which follows shows that points G, H, J, and K do fall on a straight line with a slope equal to that of line A-B. It should be emphasized that the slope of line A-B is equal to the rate constant for decomposition at constant temperature and is the parameter required for the Arrhenius plot. The slope of line A-B can be set equal to

$$\frac{R - S}{T_7 - T_2} = k \qquad \text{(Eq. 1)}$$

In coordinate notation, point G has been written as (Y, T_2) while point K has the coordinates of (Q, T_7). These points can be denoted also as ($S-X$, T_2) and ($R-Z$, T_7), respectively, where X and Z are concentration units such that

$$\ln S - \ln X = \ln Y \qquad \text{(Eq. 2)}$$

$$\ln R - \ln Z = \ln Q \qquad \text{(Eq. 3)}$$

For first-order and pseudo first-order rate mechanisms, the change in the logarithm of concentration is independent of initial concentrations and dependent only on the time interval during which the reaction occurs. This is true regardless of the temperature at which the reaction takes place. Consequently, if the rate of cooling from oven to room temperature is assumed to be equal for all samples, all samples will show an equal and constant change in the logarithm of concentration during the cooling step. Thus, the fall of X units in the logarithm of concentration from S to Y during the cooling of sample 1 will equal the fall of Z units in the logarithm of concentration from R to Q during the cooling of sample 4. Therefore, X and Z are equal to each other. Points G and K can now be expressed as ($S-X$, T_2) and ($R-X$, T_7), respectively. The slope of line G-K therefore becomes

$$\frac{(R - X) - (S - X)}{T_7 - T_2} = \frac{R - S}{T_7 - T_2} \qquad \text{(Eq. 4)}$$

This slope is identical to that for line A-B as shown in Eq. 1.

By repeating this procedure, it can be established that lines G-H, H-J, and J-K also have slopes equal to that for line A-B. Since G-H, H-J, J-K, and G-K can be shown to have the same slope and have points in common, points G, H, J, and K must be on the same line.

Individual assay points collected after sample decomposition at elevated temperatures do not recognize the errors introduced by heating and cooling lags. The foregoing theoretical analysis demonstrates however that the slope of the line drawn through these points represents, without error, the rate constant of decomposition desired for the Arrhenius plot.

EXPERIMENTAL

In order to illustrate the validity of the preceding theoretical treatment, two experiments were performed on a simple dye system degrading according to apparent first-order kinetics. The color stability at 87 ± 0.5° of aqueous solutions of FD&C Blue No. 2 (0.002%) in phosphate buffer, pH 6.72, was investigated. Residual color was measured spectrophotometrically at 610 mμ with a Cary model 11 recording spectrophotometer. Beer's law held for the dye in buffer solutions over the concentration range studies. Two different procedures were used to bring the color solutions to thermal equilibrium.

Experiment A—Instantaneous Heating and Cooling.—A 2.0-ml. aliquot of a concentrated stock solution of the dye at 25° was pipetted into 300 ml. of buffer solution preheated to 87 ± 0.5° and maintained at this temperature in a constant temperature bath. The solutions were mixed for 20 seconds to obtain uniform color distribution. The temperature of the buffer solution was not lowered by the

addition of the dye solution, thereby indicating instantaneous attainment of thermal equilibrium. At selected time intervals, 25-ml. aliquots of the solution were removed from the reaction mixture and quickly transferred to a glass-stoppered 50-ml. Erlenmeyer flask. These flasks were placed immediately into a dry ice-acetone bath at −67°. Cooling of the sample to 25° was obtained within 20 seconds by this procedure. Absorbance measurements were performed at 610 mμ for residual color. The change in the observed absorbance with time for this system is plotted in Fig. 3 as line *A*.

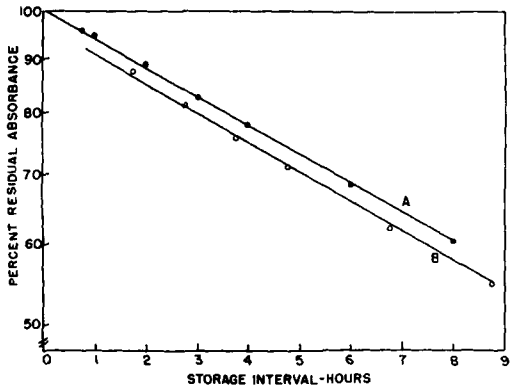


Fig. 3.—Apparent first-order plots for loss in absorbance of FD&C Blue No. 2 aged at 87°. Curve A, instantaneous heating and cooling; curve B, heating lag, 45 minutes, cooling lag, 105 minutes.

Experiment B—Slow Heating and Cooling.—This experiment was designed to introduce significant lags in both the heating and cooling of the dye solution. For this work a 2-ml. aliquot of concentrated stock solution of dye at 25° was pipetted into 300 ml. of buffer solution at 25°. The solutions were mixed to give uniform color distribution. The mixed solution was then placed in a constant temperature bath and heated to 87°. The solution required 45 minutes to reach an equilibrium temperature of 87 ± 0.5°. Periodically, 25-ml. aliquots of the solution were removed from the reaction flask and placed into a 50-ml. glass-stoppered Erlenmeyer flask. These samples were stored at room temperature to cool. A period of 105 minutes was required for the samples to reach 25°. Absorbance measurements were then taken. The data collected in this study are presented in Fig. 3 as line *B*. Storage intervals plotted in Fig. 3 were measured from the time samples were placed in the elevated temperature bath to the time they were removed.

DISCUSSION AND CONCLUSIONS

From the data shown in Fig. 3 it is evident that the rate of color loss follows first-order kinetics for experiment A (instantaneous heating and cooling). The rate constant at 87° for the isothermal reaction is expressed by the slope value of line *A* and is equal to 4.21×10^{-2} hr.$^{-1}$. It is also possible to read a $t_{80\%}$ value (3.6 hours) and other $t_{\%}$ values directly from the curve. These parameters often are used as convenient indexes of decomposition rate in the Arrhenius plot.

The degradation observed in experiment B (slow heating and cooling) also follows first-order kinetics once isothermal storage conditions are achieved (after 45 minutes). The data obtained in this experiment lie on a straight line (line *B*) which is parallel to that for the isothermal reaction. The rate constants as measured by the slopes of the lines therefore are equal. The experimental study thereby confirms the theoretically derived conclusion that valid rates of degradation can be measured directly from assay data which may be subject to (absolute) error as a result of heating and cooling lag times. Results obtained from customary stability testing of pharmaceutical dosage forms stored in constant temperature ovens therefore can be used with confidence to give accurate estimates of product stability.

Although data collected in such studies will give linear relationships with correct slope, the line constructed through the individual data points may be displaced from that obtained under isothermal conditions. Extrapolation of the decomposition data generally will not give a concentration intercept value equal to the experimentally determined concentration at zero time (100%). The rate of decomposition therefore cannot be correctly expressed in terms of $t_{\%}$ values which assume a linear relationship whose intercept on the concentration axis is equal to 100% (zero % decomposition).

When heating and cooling lags occur during the course of a stability study at elevated temperature, rate constants must be obtained from the slope of decomposition line. This slope should be calculated directly from the coordinates of two points which actually fall on the line. Although a high level of confidence is generally placed on the initial (zero time) assay value of the product, this value should not be employed in establishing the rate of decomposition.

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Acid Dissociation Constants of Phenylalkanolamines

By S. RIEGELMAN, L. A. STRAIT, and E. Z. FISCHER†

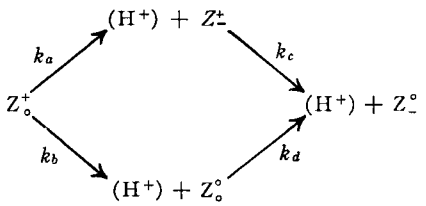
The acid dissociation constants of phenylephrine, synephrine, and tyramine were investigated by the new spectrophotometric method of Edsall. Epinephrine was studied by a titration and complexation method. It is concluded from analysis of the spectral data that the phenolic function is a stronger acid than the alkylammonium group. This is contrary to the accepted assignment of acid strengths to these acid functions. The spectrophotometric data were analyzed for the individual microscopic dissociation constants and the degree of dipolar character is reported.

THE ACCURATE evaluation of the dissociation constants of phenylalkanolamines is important to an understanding of their association and chelation reactions, their stability, and their comparative biological activity. Phenylalkanolamines may be considered initially as substituted phenols and compared to the acid strength of *o*-, *m*-, and *p*-cresol, whose dissociation constants are 10.32, 10.09, and 10.27, respectively (1). In contrast, the acidic dissociation constants of primary and secondary phenylalkylammonium groups vary from 9.8 to 10.8. Alkanolamines are somewhat stronger acids, i. e., ethanolamine has a pKa of 9.44 (2).

By analogy to these compounds, one would consider the first ionization step during the titration of a phenylalkanolamine salt to be related to the amine while the overlapping second dissociation step would be assigned to the phenol. However, evidence will be reported in this paper which will prove that the phenol group in these compounds is a stronger acid than is the alkanolammonium group.

It must be noted also that the ratio of the two successive dissociation constants obtained by pH titration may be as small as tenfold. A conventional titration, in which pH is determined as a function of the mean number of protons removed from the compound by the addition of hydroxyl ions, is not easily analyzed when the dissociation constants overlap. It is particularly difficult when the ratio of the constants for the two successive dissociation steps is 100 or less. Even when the ratio of the first dissociation constant to the second is as high as 500, one of the constants cannot be evaluated without correcting for the effect of the other, inasmuch as some simultaneous dissociation occurs. Bates (3) has stated that, under these conditions, accurate values may be unobtainable by the usual experimental procedures and has made use of the Speakman method (4) to analyze the data.

When both the phenolic and the alkylamine groups are found in the same molecule, they will not coexist without a degree of internal neutralization. Therefore, the compounds will be partly dipolar in character, depending on the relative strengths of the individual groups. At varying acidities, the molecules will exist in four different species. These may be represented as shown below



where Z^+_o = the phenylalkanolammonium ion, Z^- = the phenolate ion, and Z^+_- and Z°_o = the dipolar ion and uncharged molecule, respectively. The individual stages in the ionization result in individual or microscopic dissociation constants. These are noted as k_a, k_b, k_c, and k_d in the above diagram. The macroscopic or titration constants K_1 and K_2 bear a definite relation to the microscopic constants[1]

$$K_1 = k_a + k_b \qquad \text{(Eq. 1)}$$

$$K_2^{-1} = k_c^{-1} + k_d^{-1} \qquad \text{(Eq. 2)}$$

$$K_1K_2 = k_ak_c = k_bk_d \qquad \text{(Eq. 3)}$$

The assignment of K_1 or K_2 as determined from a titration of a compound to their corresponding dissociation function may be difficult on the basis of this information alone.

Spectrophotometry offers an alternative approach to this problem. It requires that one of the acidic groups in the molecule shall have an

Received September 12, 1960, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

Accepted for publication March 8, 1961.

Presented to the Scientific Section, A. PH. A., Washington, D. C., meeting, August 1960.

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[1] All the constants listed in the foregoing equations are acid dissociation constants. The existing literature citations are by no means uniform in the assignment of symbols for the microscopic acid dissociation constants. We have chosen to use the subscripts *a* to *d* rather than Arabic or Roman numerals to denote the different constants. It should be pointed out, however, that k_b represents an acid dissociation constant for the reaction shown in the diagram given above.

absorption spectrum distinguishable from its conjugate base. This is the case with the phenylalkanolamines. The phenol peak of the three phenylalkanolamines studied has an adsorption maximum in the region 2,720 to 2,750 Å. and shifts to 2,900 to 2,930 Å. upon complete conversion to the phenolate form. The molar extinction coefficient at 2,940 to 3,000 Å. is negligible for the forms in which the OH group is unionized, whereas it is large in the forms in which $-OH$ has been converted to the anion ($-O^-$). See Fig. 1. At pH values below 12.0, there are two forms in which the $-O^-$ exists, i. e., Z^+_- and Z°_-.

Edsall has shown that it is possible to obtain the individual constants for tyrosine by appropriate analysis of the ultraviolet absorption spectra over a wide range of pH values (5). He defined a function α_{OH}, which represents the fraction of all the phenolic hydroxyl groups in the solution which are ionized

$$\alpha_{OH} = \frac{\text{concn. of all molecules with ionized OH}}{\text{concn. of all molecular species}} = [Z^+_-] + [Z^\circ_-]/[Z^+_-] + [Z^\circ_-] + [Z^\circ_\circ] + [Z^+_\circ] = \frac{k_a(H^+) + k_ak_c(H^+)^2}{1 + (k_a + k_b)/(H^+) + k_ak_c/(H^+)^2} \quad \text{(Eq. 4)}$$

Edsall further defined M_{OH}

$$M_{OH} = \frac{(H^+)\alpha_{OH}}{(1 - \alpha_{OH})} = \frac{k_a(H^+) + k_ak_c}{(H^+) + k_b} \quad \text{(Eq. 5)}$$

or from Eq. 3

$$M_{OH} = \frac{k_a(H^+) + k_bk_d}{(H^+) + k_b} \quad \text{(Eq. 6)}$$

or in the logarithmic form

$$pM_{OH} = pH + \log(\alpha_{OH}/1 - \alpha_{OH}) \quad \text{(Eq. 7)}$$

$$= -\log \frac{k_a(H^+) + k_bk_d}{(H^+) + k_b} \quad \text{(Eq. 8)}$$

It is apparent from Eqs. 7 and 8 that a plot of pM_{OH} *vs.* α_{OH} will extrapolate to pk_a when $\alpha_{OH} = 0$ ($[H^+] \rightarrow \infty$) and to pk_d when $\alpha_{OH} = 1$ ($[H^+] \rightarrow 0$).

Once pk_a and pk_d are known, it is possible to obtain pk_b from the best fit to the experimental data. From Eq. 7, one will note that at $\alpha_{OH} = 0.5$, $pM_{OH} = pH$. Substituting this value for pM_{OH} in Eq. 8 and solving for k_b gives

$$k_b = \frac{(H^+)[k_a - (H^+)]}{(H^+) - k_d}, \text{ at } \alpha_{OH} = 0.5 \quad \text{(Eq. 9)}$$

Alternatively, a second approach leads to the value of k_b. Considering the value of α_{OH} corresponding to the point at which $pM_{OH} = (pka + pkd)/2$, then for this particular value of $M = (k_ak_d)^{1/2}$ we obtain from Eq. 6

$$pk_b = \log \frac{\alpha_{OH}}{1 - \alpha_{OH}} + pk_a \quad \text{(Eq. 10)}$$

The above calculation is exact if the molar extinction coefficient of the two phenolate forms Z^+_- and Z°_- are identical at the wavelengths chosen for the analysis. At the worst, it is a close approximation for the phenylalkanolamines. The same applies to the free phenol forms Z°_+ and $Z^\circ_\circ$, but is less significant since they make a very small contribution to the total results. If either assumption were incorrect, repeating the analysis at several different wavelengths should result in different values for the derived constants. The data obtained for the three phenylalkanolamines herein reported met this critical test.

EXPERIMENTAL

Apparatus.—Melting points were determined on a Kofler melting point apparatus. Polarimetric measurements were done on a Rudolph research polarimeter. The pH measurements were made on a Beckman model G pH meter. The ultraviolet absorption spectra were measured on a Cary model 11 recording spectrophotometer.

Materials.—l-Phenylephrine hydrochloride U. S. P. (Gane and Ingram Co., N. Y.) was used; m. p. 134–144°; specific rotation $[\alpha]^{25}_D$ −43°. Tyramine monohydrochloride (Nutritional Biochemicals Corp.), m. p. 269°. Synephrine tartrate was furnished by Dr. M. L. Tainter of Sterling Winthrop Research Institute. In several instances, the free bases were recovered, recrystallized, and used for the studies. There appeared to be no difference in extinction coefficients relative to the original salts. Epinephrine base (Desmo Chemical Corp.) meeting U. S. P. specifications was used as received.

Procedure.—Stock solutions were freshly prepared and diluted to approximately 3×10^{-3} to $4 \times 10^{-4} M$. All solutions were adjusted to a constant ionic strength of 0.1 *M* by the addition of potassium chloride. The recording spectrophotometer was carefully adjusted to zero absorbance prior to the determination and verified regularly between determinations. The instrument was carefully adjusted to wavelength and to zero absorbance at 3,500 Å. where the solutions were optically transparent.

The solutions were neutralized directly in the cell by the addition of a 5 *M* solution of acid or base using a micropipet. A range of pH values from less than 4.0 to greater than 12.5 was covered. The Beckman model G pH meter with a type E-2 electrode was checked for calibration, using the appropriate primary or secondary buffer standard recommended by the National Bureau of Standards, immediately before and after each pH reading. Complete neutralization of the sample modified the volume less than 0.5%. In all instances, the pH of the solution being studied was determined directly in the cell before and after analysis of its U. V. ab-

sorption. The values rarely differed more than 0.02 pH unit. It was found necessary to add a buffer to the synephrine solutions in order to stabilize the pH values in the intermediate range. Tris-(hydroxymethyl)-aminomethane was used as the buffer at a concentration of approximately tenfold of the drug concentration. The buffer is optically transparent in the region of study. In the acidic range, a more concentrated solution of the phenylalkanolamine was used when necessary in order to increase the accuracy of the determination of the absorbance of the solutions predominately containing the free phenol forms. However, in order to calculate the fraction in the phenolate ion form, the solution had to be brought to the pH >12.5. A carefully calibrated spacer was used to reduce the path length in order to obtain the absorbance of the highly absorbing phenolate ion form. In order to reduce the effect of oxidative changes, all solutions were freshly prepared just prior to the study. The solutions remained colorless throughout the study. The absence of oxidative changes was proved by checking whether or not the original spectrum was recovered by conversion of the alkaline solution back into the free phenol form.

Calculations of pM_{OH} values were made at three wavelength values in the region of 2,920 to 3,000 Å. where the extinction coefficient of the free phenol form is minimal, but the phenolate forms result in a high extinction. Within experimental error, the results reported are independent of the wavelength selected.

The titrations of epinephrine in the presence and absence of boric acid were run in a four-neck, round-bottom flask of 500-ml. capacity. The epinephrine base was dissolved with a slight excess of hydrochloric acid adjusted to 0.1 *M* with potassium chloride, and titrated with carbon dioxide-free potassium hydroxide. The titrating solution and the flask were flushed free of dissolved oxygen by passing oxygen-free nitrogen through the solution. The nitrogen flushing was continued during the run. Only an insignificant discoloration appeared at the end of the titration. The nitrogen was freed of residual oxygen by passing the gas through a gas purification train containing vanadous sulfate (6) and 2,6-anthroquinone disulfonate as oxygen getters.

RESULTS AND DISCUSSIONS

Figure 1 illustrates a typical determination of ultraviolet absorption data of phenylephrine. It is generally recognized that binary mixtures of compounds will possess isosbestics at points of intersection when only the mole fraction is varied. Examination of the graphs indicate that the solutions do not possess perfect isosbestic points; they have slightly varying points of intersection. These results were demonstrated not to arise from errors in the measurement procedure. This indicates that there are three or more absorbing species present in the majority of the solutions studied. Imperfect isosbestic points may be seen in the published data of Edsall, *et al.*, on glycyl-L-tyrosine at various pH values (7); however, they did not comment on the significance of this phenomenon.

The ultraviolet absorption data were converted to the appropriate form according to Eq. 7. The results are plotted in Fig. 2 in the form of pM_{OH}

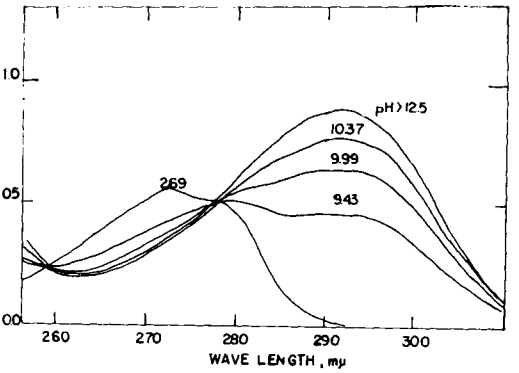


Fig. 1.—Ultraviolet absorption spectrum of phenylephrine hydrochloride at various pH values; temperature 22–23°; ionic strength 0.10.

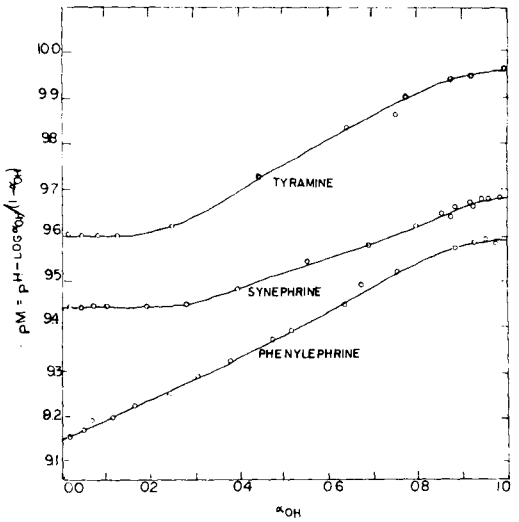


Fig. 2.—Values of pM_{OH} as a function of α_{OH} for phenylephrine, synephrine, and tyramine, respectively (see Eqs. 7 and 8).

vs. α_{OH}. As discussed above, the extrapolation to $\alpha_{OH} = 0$ and $\alpha_{OH} = 1$ results in the determination of pk_a and pk_d, respectively. These assignments are explicit from the equations listed earlier. Equations 9 and 10 were both used for the estimation of pk_b. The values were in good agreement. The solid line drawn through the points is the result of the substitution of the three microscopic constants into Eq. 8. Modification of the experimental microscopic constants by ±0.02 results in theoretical curve which deviates markedly in the sensitive portion of the graph.

Table I summarizes the data on the macroscopic and microscopic acid dissociation constants for the compounds studied. Both Fig. 2 and Table I indicate that the *meta*-substituted compound possesses ionization properties which differ from those of the *para*-substituted compounds. It is well recognized that several resonant forms of phenols exist with centers of high electron density at the *ortho* and *para* positions relative to the phenol group. Thus, phenylephrine with its *meta* substitution possesses carbons of increased negative charge density on

TABLE I.—NEGATIVE LOGARITHMS OF IONIZATION CONSTANTS

Constant[a]	Compound: Phenylephrine	Synephrine	Tyramine
pK_1	8.77	9.29	9.37
pK_2	9.84	10.24	10.70
pk_a	9.14	9.44	9.59
pk_b	9.02	9.84	9.66
pk_c	9.47	10.09	10.03
pk_d	9.59	9.69	9.97
$Z_-^+/Z_o^{\circ b}$	0.76	2.51	1.18

[a] Estimated error = ±0.02. $k_a/k_b = k_c/k_d = Z_-^+/Z_o^\circ$.

either side of the alkanolamine chain, while synephrine does not. An atomic model of the compounds indicates that the secondary amine group can make contact with these carbon atoms. The values listed for the pk_a, pk_b, and pk_c for phenylephrine show increased acidity relative to the analogous *para*-substituted compounds. In contrast the values for the pk_d of phenylephrine and synephrine show a smaller difference, more analogous to that shown by *m*-cresol when compared with *p*-cresol.

Table I includes the macroscopic acid dissociation constants as calculated by Eqs. 1 and 2. A similar spectrophotometric study was conducted in 1954 by Lewis (8) who determined the ionization constants of sympathomimetics including the drugs used in this study. He calculated the phenolic dissociation constants, pK_1, on the basis of an assumption that only one species contributed to the phenolate spectrum or to the free phenol spectrum. Our data, however, show the failure to produce unequivocal

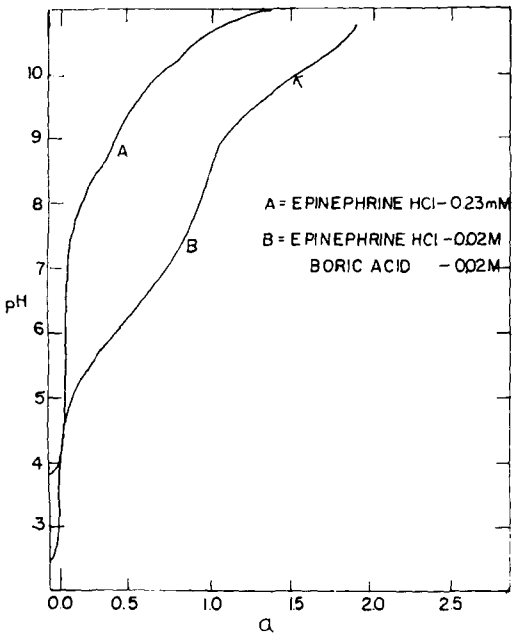


Fig. 3.—Titration of epinephrine hydrochloride with KOH, in the absence (*A*) and presence (*B*) of equimolar boric acid; ionic strength = 0.1; "a" denotes moles of base used per mole of epinephrine added.

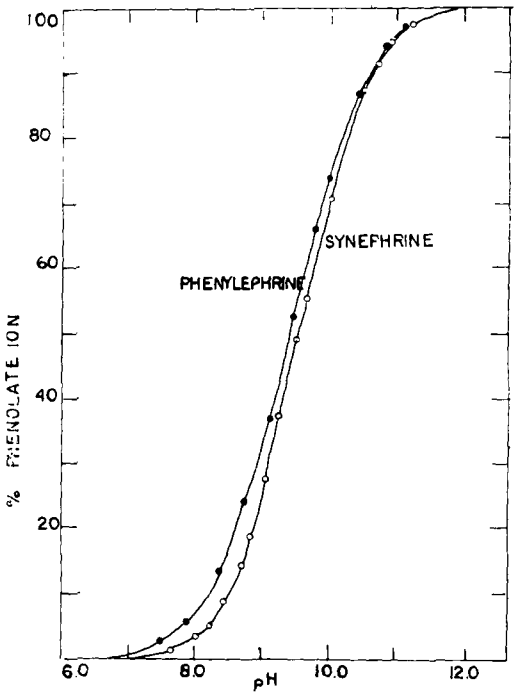


Fig. 4.—The degree of ionization of the phenol group of phenylephrine and synephrine (α_{OH}) as a function of pH.

isosbestic points in these systems. Therefore, the assumption of Lewis is unwarranted for these compounds and probably for the other dibasic compounds so calculated. Lewis then used the value obtained to estimate the pK_2 of the amino group from titration studies. Although both pK's are quantitatively inexact, they do confirm our conclusion that the conjugate acid of the amine is a weak acid relative to the phenol group. For experimental and theoretical reasons discussed in this paper, the acid dissociation constants (titration constants) determined by Tuckerman, *et al.* (9), using the approximation method of Parke and Davis (10) are invalid relative to their assignment of the particular dissociation constant to the amino group as well as to the relative accuracy of calculated constants.

The complication ensuing in the assignment of the titration acidity constants is illustrated in Fig. 3, the plot of our titration data of epinephrine in the absence (*A*) and presence (*B*) of equimolar concentration of boric acid. Boric acid is known to form chelates with adjacent or *cis* oriented hydroxy groups. In this manner, it is possible to block the normal ionization characteristics of phenolic groups. The first dissociation step in curve *B* of Fig. 3 is believed to be due to the second reaction represented below

$$H\,B + E \rightleftarrows H\,B\,E$$

$$H\,B\,E \rightleftarrows H^+ + B\,E^-$$

where HB = boric acid and E = epinephrine. Since the hydroxyl groups are blocked and no excess of boric acid is present, the second equivalent of base

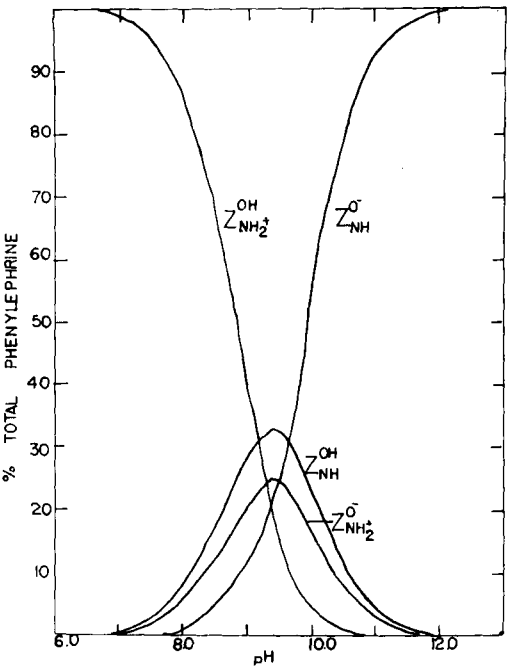


Fig. 5.—Relative concentrations of different microscopic forms of phenylephrine at different pH values.

which is utilized in the reaction must be assigned to the ionization of the secondary alkylammonium group of epinephrine. This leads to a pK_- of approximately 9.9. Tuckerman, *et al.*, incorrectly assigned a value of 8.55 to this functional group in epinephrine. Our titration value for epinephrine is in good agreement with the value of Lewis (7). It should be noted also that the pK's obtained by Albert (11) for tyramine of $pK_1 = 9.3$, $pK_2 = 10.9$ are in fair agreement with our results as shown in Table I considering the ionic strength effect and the differences in the method of calculation.

Table I also includes the ratio of the number of molecules in the dipolar ion form to the number in the uncharged form. This ratio gives no indication of the absolute amount of any of the species. However, these may be derived by further analysis of the absorption spectra data according to the procedures used by Benesch and Benesch (12). Figure 4 represents the degree of ionization of the phenol group (α_{OH}) of phenylephrine and synephrine as a function of pH, which is the sum of $Z^{\pm}_{-}$ and Z°_{-}. The data in Fig. 4, along with the fact that the ratio of the dipolar to the uncharged molecule is constant at all pH values, provide two simultaneous equations and to solve them for the relative concentrations of the different microscopic forms at different pH values. Figure 5 illustrates such an analysis of the data found for phenylephrine. It should be noted that the compound possesses significant concentrations of all species in the pH range from 8 to 10.5. At pH 9.35 where the dipolar ion form reaches its maximum concentration, the solution contains approximately 25% $Z^{\pm}_{-}$, 33% $Z^{\circ}_{\circ}$, 21% Z°_{-}, and 21% $Z^{+}_{\circ}$. It has been assumed, on occasion, that the evaluation of the ratio of $Z^{\pm}_{-}/Z^{\circ}_{\circ}$ was identical with a complete calculation of the per cent of the dipolar ion form. Such as assumption would lead to a conclusion that phenylephrine was 43% in the dipolar form. The more definitive analysis as given above shows that phenylephrine possesses a maximum of 25% in the dipolar ion form and this only at a pH of 9.35.

Postulation as to the significance of the individual microscopic dissociation constants on the stability and on the association or chelate formation properties of phenylalkanolamines will be left to a later publication.

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Ion Exchange Resin Salts for Oral Therapy I

Carbinoxamine

By DAVID A. SCHLICHTING

Six new carbinoxamine cation exchange resin salts have been prepared. The degree of dissociation under simulated gastrointestinal tract conditions has been studied. It has been found that salts formed with resins having a pKa less than 5.2 are most adaptable to slow elution and, hence, sustained release of carbinoxamine. A more detailed investigation of salts of polystyrene sulfonic acids was made. Conditions regarding resin loading, per cent of cross linking of resin, efficiency of exchange, and the presence of added free acid resin were considered for attaining slow dissociation characteristics. Pharmacological tests of antihistamine activity are described. A new *in vivo* method of determining total drug release from an ion exchange salt is described and related to an *in vitro* method. The preparation of dosage forms and their stability is briefly discussed.

MOST ANTIHISTAMINIC agents have a central nervous system side effect. Frequently, a parasympatholytic effect is associated with them. The majority of these agents have a duration of action of about 4 hours or less (1). In the treatment of many conditions requiring antihistamines, a sustained antihistaminic effect is sought. If near optimal release of medicament can be achieved, then sustained drug blood levels and a reduction in side effects would be anticipated. Consequently, the possibility of achieving controlled and sustained release of the antihistamine carbinoxamine, 2-[*p*-chloro-α-(2-dimethylaminoethoxy)benzyl]pyridine, was investigated. A study was made of insoluble cation exchange resin salts, their formation, and dissociation under *in vitro* simulated gastrointestinal tract conditions and an *in vivo* test. Carbinoxamine maleate is a water-soluble, readily absorbed amine salt of proved clinical utility as an antihistaminic agent. A 4 to 8-mg. dose gives an effective clinical response and is recommended by the manufacturer to be taken three to four times a day.

Use is made of insoluble, synthetic cation exchange resins for salt formation and release-rate evaluation. They allow for utilization of numerous variables. Resins of varying functional anions, pKa values, cross linkages, and particle sizes are available. These variables are utilized in the following experiments in an attempt to prepare salts of carbinoxamine that release the drug slowly.

EXPERIMENTAL

Materials.—The insoluble, synthetic cation exchange resins used are described in Table I with information from the manufacturers as to their characteristics.[1] Future reference to these resins by capital letter will refer to Table I.

Resin Salt Formation.—Preparation of the carbinoxamine salts of the various resins was accomplished in the usual manner employing either batch or column processes (2). Carbinoxamine, its maleate, and its hydrochloride were used in aqueous and water-alcohol solutions in concentrations varying from about 1 to 20%. Quantities of carbinoxamine varying from two and one-half times the theoretical capacity to one-fourth of the capacity were used. Figure 1 exemplifies the rate of salt formation and is typical of this specific reaction as well as others studied.

Assay.—Assay of the various salts formed was accomplished by column elution with 5 to 10% hydrochloric acid. Elution with 2–300 ml. of acid over about a 24-hour period of time was found to be adequate. A Beckman spectrophotometer, model DU, was used for assay, reading at 263 mμ. Minor modifications were necessary when the resin salt was formulated in tablet and suspension dosage forms. Table II gives the results of the assays of carbinoxamine salts of the resins described in Table I.

Release Rates.—Time-release relationships were studied by subjecting a 40–100 mg. sample of the resin salt to 75 ml. of U.S.P. simulated gastric fluid without pepsin for one hour at 37.5 ± 3°. After 1 hour, the eluate was removed and assayed and was replaced by 75 ml. of U.S.P. simulated intestinal fluid without pancreatin. Aliquots, 5 to 10 ml., of this latter fluid were removed and assayed periodically and fresh replacement samples were added. The method is of the type described by Chaudhry and Saunders (3) as the replacement closed tube method.

The test apparatus used for time-release rates utilized 2.2 × 9-cm. glass tubes. They were fitted with 100-mesh stainless steel screens at the bottom and hooks at the top. Test samples were placed in such tubes. The tubes were hooked onto the arm of a U.S.P. device for raising and lowering tubes (4).

Received April 19, 1961, from McNeil Laboratories, Inc., Philadelphia, Pa.
Accepted for publication May 8, 1961.

[1] The materials and information were kindly supplied by Chemical Process Co. (Duolite), Dow Chemical Co. (Dowex), National Aluminate Corp. (Nalcite), and Rohm and Haas Co. (Amberlites).

TABLE I.—DESCRIPTION OF INSOLUBLE, SYNTHETIC ION EXCHANGE RESINS STUDIED

Code	Name of Resin	Chemical Description Main Constituent	Approx. % Cross Linkage with Divinyl Benzene	Particle Size Range, U. S. Std.	Approx. Exchange Capacity, Meq./Gm. Dry Basis	Approx. pK, 1 *M* NaCl
A	Amberlite IR-120	Polystyrene sulfonic acid	8–10	30–40	5	1.3
B	Dowex 50 W X 8	Polystyrene sulfonic acid	8	50–100	5	1.3
C	Dowex 50 W X 12	Polystyrene sulfonic acid	12	50–100	5	1.3
D	Duolite C-3 (H)	Phenol formaldehyde sulfonic acid	None	16–50	3 (Sulfonic)	1.3
E	Amberlite XE-89 (H)	Poly acrylic acid	5–7	16–50	6–7	5.2
F	Amberlite XE-96 (H)	Poly methacrylic acid	4–6	16–50	10	6.0
G	Amberlite XE-64 (H)	Poly methacrylic acid	4–6	100–325	10	6.0
H	Amberlite EM-267 (H)	Poly acrylic:sulfonic acids in 7:3 ratio	11.5	16–50	$-CO_2H$ 9.8 $-SO_3H$ 2.1	5.2 1.3
I	Nalcite X-219 (Na)	Sodium polystyrene phosphonate	...	16–50	5–6	3.2 7.6

TABLE II.—ASSAY AND RELEASE OF CARBINOXAMINE FROM VARIOUS RESIN SALTS

Salt of Resin Tested	% Carbinoxamine in Salt	Total Release Rate, % 1 hr.	2 hr.	4 hr.	5 hr.	6 hr.	7 hr.	8 hr.
A	21.9	5	32	50	..	58	..	61
B	23.6	2	25	40	..	44	..	47
C	18.7	1	19	30	..	35	..	36
D	8.4	19	45	56	..	61	..	62
E	54	99	99	99	..	99	..	100
F	21.4	89	92	91	..	91	..	..
G	24.2	90	95	94	..	96	..	..
H	9.6	37	40	49	56	56	56	..
I	32.5	72[a]	76	83	86	87	..	..

[a] A 0.5-Gm. sample studied for 4½ hours in gastric fluid and 1½ hours in intestinal fluid.

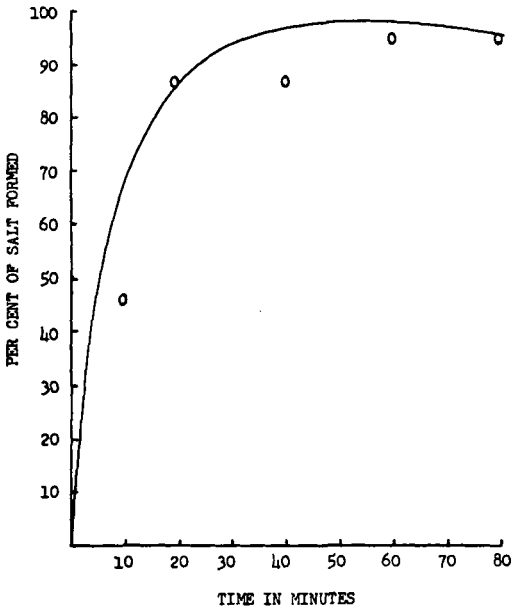


Fig. 1.—Reaction rate at room temperature between a 2.8% aqueous carbinoxamine maleate solution, equivalent to 25% of the theoretical capacity of the resin, and resin A.

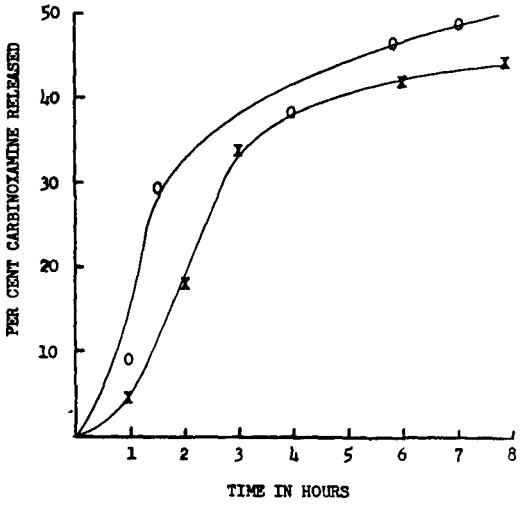


Fig. 2.—Rate of dissociation of two salts assaying 25.0 and 25.6% carbinoxamine, respectively. O, Surface area = 1,463 mm.²/100 mg.; X, surface area = 758 mm.²/100 mg.

The tubes containing samples were allowed to move up and down in individual 4 × 15-cm. test tubes containing 75 ml. of eluant maintained at 37.5 ± 3°. Table II describes the release rates of the various resins described in Table I.

In 5 morphine-chloralose anesthetized dogs, a 0.7 mg./Kg. dose of carbinoxamine, given intraduodenally as the maleate salt, showed maximal response to intravenous histamine in an average time of 33 minutes. The elapsed time from the average maximal response until histamine response returned to one-half the control response averaged 101 minutes. In 4 morphine-chloralose anesthetized dogs, a 2 mg./Kg. dose of carbinoxamine on resin, intra-

duodenally, showed maximal response to intravenous histamine in an average time of 74 minutes. The elasped time from the average maximal response until histamine response returned to one-half the control response averaged one 106 minutes.[2] Assuming about 50% dissociation of the resin salt, the prolonged protection is significant.

An *in vivo* study of total release of carbinoxamine from polystyrene sulfonic acid in a healthy adult male was accomplished. A sample of resin A salt, shown in Fig. 2 as X—X, containing 33 mg. of carbinoxamine was ingested 5 hours after the evening meal and 8 hours before the morning meal. A portion was recovered from the feces about 24 hours later and was isolated and assayed for carbinoxamine remaining on the resin. It was found that this dose released 53% of the carbinoxamine in a normal gastrointestinal tract compared to 44% released by the *in vitro* test method reported.

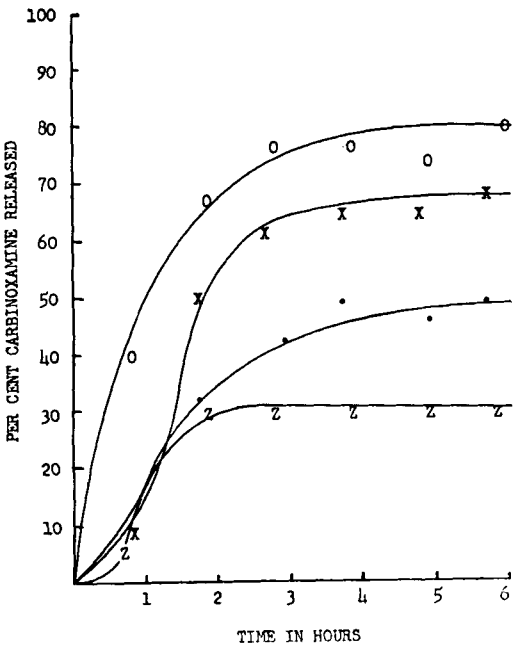


Fig. 3.—Rate of dissociation of smaller than 80-mesh samples. O, Sample 65% converted to salt form; X, same salt plus equal weight of resin A acid; Z, same salt plus three times the weight of resin A acid; ●, sample 47% converted to salt form.

RESULTS AND DISCUSSION

Several variables were shown to have important effects on dissociation of the resin salts as reported in Table II. Resins having a pKa 5.2 or greater release carbinoxamine too rapidly for sustained action, e.g., resins E, F, and G. Resins having a pKa less than 5.2 release carbinoxamine in a more desirable manner for sustained release effect, e.g., resins A, B, C, D, and H. For those resins having two pK values, the overall effect is between the two values. Resin A gives time-release characteristics closest to a straight line function and reacts efficiently with carbinoxamine. Increase in cross linkage of the polymers reduces both the efficiency of salt formation and of dissociation. Figure 2 shows the effect of surface area on release characteristics. Doubling of the surface area increased the dissociation *in vitro* by about 5% in this test. Figure 3 shows that partial loading of resin A or introduction of acid resin A in the presence of resin salt A gives results similar to those reported by Chaudhry and Saunders (3). However, the salt plus equal weight of resin releases more than expected.

Tablets were formulated and it was found that the salt of resin A, ground to smaller than 40 mesh, could be compressed well when aluminum hydroxide or dicalcium phosphate was used as the main tablet diluent and gelatin solution was used in granulation. Such tablets, colored with pigments or other tablets coated with sugar, showed no alteration in assay or release characteristics on storage for 3 months at about 40°.

It was found that the salt of resin A, ground to smaller than 80 mesh, could be suspended well in tragacanth or Carbopol 934[3] sols. The effect of pH on release was studied and indicates that a weak ionic aqueous suspension would release little or no carbinoxamine between pH 3 and 5. Assay and release characteristics of suspensions showed no alteration on storage for 3 months at about 40°.

SUMMARY AND CONCLUSIONS

Six new insoluble synthetic cation exchange resin salts of carbinoxamine have been prepared and evaluated. Salt formation and dissociation were studied and polystyrene sulfonic acid was found to be superior. Retardation of dissolution occurred, however, with all salts made from resins having a pKa less than 5.2. For the preferred acid salt it was found that: particle size plays a minor role in dissolution rate, partial salt formation or the presence of added free acid markedly alters dissolution rate, and increased crosslinking of the resin reduces the dissolution rate. In anesthetized dogs it was shown that the resin salt protected against histamine for a significantly longer time than carbinoxamine maleate. A new *in vivo* test method of total release was compared with the *in vitro* test method used and they were found to be of the same order of magnitude. Tablets and suspensions of the resin salt have been prepared and found to be stable over a period of 3 months at about 40°.

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[2] The assistance of Mrs. R. G. Peterson is gratefully acknowledged.

[3] Kindly supplied by the B. F. Goodrich Co.

Peptide Studies V

Synthesis and Racemization of Some Optically Active Tripeptides of L- and D-Leucine and L- and D-Valine

By S. SHANKMAN and S. HIGA

Seven tripeptides of leucine and valine of the optical configuration L-L-D were synthesized by the mixed anhydride procedure. Configuration of each amino acid in the peptides was established bacterimetrically. Racemization was found on the internal amino acid.

IN PREVIOUS investigations of antibacterial peptides (1, 2), we found that the L-L-D structure of the 8 valine tripeptides was unique in its ability to inhibit growth of lactic acid bacteria. Synthesis of other tripeptides was desirable to determine further the relation of structure to inhibitory activity. We now report that the 7 other possible L-L-D tripeptides of leucine and valine have been synthesized via the mixed anhydride procedure (3) by the sequence Z.A.B. + C. ester → Z.A.B.C. ester. All di- and tripeptide intermediates were obtained in the crystalline state. The phthalyl valyl-valyl esters were previously reported (4) as uncrystallizable oils.

In the synthesis of carbobenzoxy-leucyl-valyl-leucine methyl ester and carbobenzoxy-leucyl-valyl-valine methyl ester, both the desired L-L-D structure and an L-DL-D mixture were isolated. The latter was formed by racemization of the central amino acid. This mixture, which melted sharply, is composed of two compounds, L-L-D and L-D-D, which crystallized together but are not optical enantiomorphs. The L-DL-D mixtures contained approximately equal amounts of L-L-D and L-D-D isomers for both the Z.leu.val.-val.ester and the Z.leu.val.leu.ester.

The optical purity of peptides has been established classically by rotation of the protected peptide after synthesis by two procedures. Several workers (5–10) have evaluated coupling procedure for racemization by using model systems with isolation of the optically active and racemic compounds. The coupling of acylamino acid to the peptide esters has been recommended to avoid racemization (11, 12). Z-blocked dipeptides may racemize during *p*-nitrophenyl ester formation (13). Racemization of an internal amino acid may occur during exposure of the peptide to base (14) during the saponification of peptide esters.

Biologic evaluation of optical purity has proved useful when the peptide can be compared to natural all L isolated material (15, 16). In one case, however, presence of D-amino acid did not destroy or prevent biologic activity (17). Enzymatic methods, employing leucine aminopeptidase (18) and chymotrypsin (19), have been used to establish optical purity after decarbobenzoxylation of peptides. Still another method for proving optical purity of peptides is the bacterimetric determination of the free L-amino acids after hydrolysis (4). Here we report the application of the bacterimetric procedure to determine the configuration of each amino acid in the free tripeptides of leucine and valine and two carbobenzoxy-tripeptide esters.

EXPERIMENTAL

Carbobenzoxy-valyl-valyl-methyl ester.—Z.L-valine (0.138 mole) (m.p. 59–60°) in cold (−10°) ether was condensed via the chlorcarbonate (3) with valine methyl ester (0.153 mole) freshly distilled. The mixture was washed with water, 0.1 N hydrochloric acid, water, 5% potassium carbonate, water, with appropriate backwashes, dried over sodium sulfate, and evaporated to dryness. The compound solidified after the residual solvent was removed *in vacuo*. The peptide was recrystallized from chloroform:petroleum ether. Yield 37.5 Gm., 75%, m.p. 109–110°. The other three protected dipeptides were similarly made. Yields and m. p. are given in Table I.

Carbobenzoxy-valyl-valine.—Z.val.val.OMe (0.088 mole) was dissolved in a minimal amount of methanol at RT and sodium hydroxide (0.096 mole) (2 *N* in water) was added. After 4 hours water was added and the methanol was removed *in vacuo*. The unsaponified residue was removed by filtration. The aqueous filtrate was treated with about 1 Gm. of activated carbon, filtered, and acidified with 1 *N* hydrochloric acid to Congo red. The Z.val.val was extracted into ethyl acetate, washed with water, dried, evaporated to dryness, and recrystallized from chloroform:petroleum ether. Yield 26.9 Gm., 88%, m.p. 140–141.5°. The remaining Z.dipeptides were similarly made.

Received April 20, 1961, from Pasadena Foundation for Medical Research, Pasadena, Calif.
Accepted for publication May 24, 1961.
This work was supported in part by Grants CY-3609 and CY-4318 of the National Institutes of Health.
The authors wish to thank Mrs. V. Gold for assistance with the analyses.

TABLE I.—CARBOBENZOXY DIPEPTIDES OF L-LEUCINE AND L-VALINE

Compound	Z. Dipeptide Ester			Z. Dipeptide		
	Yield, %	M.P., °C.[a] Found	Lit.	Yield, %	M.P., °C. Found	Lit.
Leu.leu	70	95–96	97–98.5(20)	80	114–115	114–116(21)
Val.leu	60	103–104	112(22)	89	137–137.5	143(22)
Val.val	75	109–110	100–103(23)	88	140–141.5	139.5–140(24)
Leu.val	70	...	...	80	109–110	109–110(25)

[a] Uncorrected.

Carbobenzoxy-tripeptide Esters.—Z.leu.leu, 2.5 Gm. (6.6 mmole) in cold ether (−5°) was condensed via the chlorcarbonate (3) with 7.3 mmole of D-valine ester freshly distilled. Some precipitation occurred on standing overnight. To the entire mass was added sufficient ethyl acetate to form one phase, and the ethyl acetate was washed with water, 0.1 *N* hydrochloric acid, water, 5% potassium carbonate, and water, with appropriate backwashes. The ethyl acetate-ether phase was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in chloroform and fractionally precipitated with petroleum ether (30–60°). The first and second crops were recrystallized repeatedly from the same solvent pair to constant melting point before any analysis. The remaining six compounds were similarly prepared unless noted. Yields and analytic data are given in Table II.

On the Z.leu.val.val.OMe and Z.leu.val.leu.OMe, the above procedure gave the sharp melting material which melted intermediate to the compounds in Table III.

Fractionation of Z.leu.val.val.OMe.—The first crop, 1.5 Gm. of product from chloroform:petroleum ether crystallization, had a wide melting range 130–150°. This material was very slowly crystallized five times from ethanol:water to yield 0.5 Gm. of material, m.p. 131–132°, which proved by analysis to be a mixture of L-L-D and L-D-D. The second crop from the chloroform:petroleum ether was obtained by evaporating the filtrate from crop No. 1 to dryness. It was recrystallized three times from ethanol:water to yield 0.4 Gm. of material, m.p. 162–163°, which proved on analysis to be chiefly L-L-D.

Fractionation of Z.leu.val.leu.OMe.—The crude tripeptide ester (3.2 Gm.) was recrystallized from methanol:water to give 2.4 Gm., m.p. 130-153°. After two recrystallizations from chloroform:hexane and one from alcohol:water, 1.0 Gm. of material, m.p. 126–127.5°, was obtained, which was by analysis a mixture of L-L-D plus L-D-D. The second crop from chloroform:hexane was obtained by evaporating the filtrate from crop No. 1 to dryness. It was recrystallized very slowly three times from ethanol:water to give 0.8 Gm., m.p. 162–164°, which proved to be the L-L-D compound.

Carbobenzoxy Tripeptides.—These compounds were saponified as above, but overnight. The methanol was reduced to one-fourth volume *in vacuo*, and water was added to precipitate completely the unsaponified starting compound. The filtrate was stripped of methanol on a rotary evaporator, extracted with ethyl ether, treated with charcoal, and acidified with 0.1 *N* hydrochloric acid to Congo red. The Z.tripeptides were filtered off and recrystallized from chloroform:petroleum ether. Ethanol:water was used only when material with wide melting range was obtained.

Tripeptide Acetates and Tripeptides.—The Z.tripeptide (0.5 Gm.) was dissolved in 30 ml. methanol and 0.1 ml. acetic acid, 2 ml. water, and 0.17 Gm. palladium oxide catalyst (26) was added. Hydrogen was passed in until carbon dioxide was no longer evolved. The catalyst was removed and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, filtered, and again evaporated to dryness. This procedure gave tripeptide acetates. The tripeptide acetates were put on a Dowex-50-X-8 column and eluted with 4 *N* ammonium hydroxide. The eluate was taken to dryness *in vacuo*.

Analytic Procedures.—*L. plantarum* was used to assay L-valine under conditions in which D-valine was inactive. *P. cerevisiae* parent (ATCC 8081) or *L. casei* was used to assay for L-leucine, since *L. plantarum* can utilize the D-form. Media and test conditions have been reported (27). The growth period was 3 days. Several commercial D-leucines had variable amounts (3–11%) of L-leucine under our conditions. The lot used for synthesis assayed 0.1–0.3% L-leucine in several trials. The D-valine employed assayed about 0.1% L-valine. The L-amino acids from Calbiochem or Mann assayed about 100% of control by bacterimetry. Possible impurities, such as tyrosine in L-leucine, were negligible. D-Amino acid in L-valine or L-leucine was not determined.

Free peptides were hydrolyzed overnight in 4 *N* hydrochloric acid, in sealed tubes, at 116°, which is conveniently maintained by refluxing *n*-butanol. Z.peptides or Z.peptide esters were treated first with hydrogen bromide in dioxane, taken to dryness, then hydrolyzed with hydrochloric acid. Hydrochloric acid treatment alone led to charring of sample and poor assays.

Rotations were measured with the Keston polarimetric attachment to the Beckman DU spectrophotometer, employing Aminco 5.00-cm. cells of 2.6 ml. volume. The technique for inhibition measurements has been described (2). All samples were autoclaved with the test medium.

DISCUSSION

The bacterimetric procedure, applied to any all L-peptide, gives (as a measure of optical purity of a given amino acid) the L-amino acid found compared with theory for that amino acid. In the L-L-D peptides described here, the bacterimetric procedure is applicable to each amino acid residue with maximum accuracy when used to determine the C-terminal amino acid with D-configuration. Any L-amino acid in the C-terminal position is determined

TABLE II.—L-L-D TRIPEPTIDES OF LEUCINE AND VALINE AND DERIVATIVES

Compound	Z.Tripeptide Ester		Z.Tripeptide		Tripeptide							
	Yield, %	M.P., °C.[a]	Yield, %	M.P., °C.	Form	Yield, %	M.P., °C.	% N Found	% N Theory	% L-Leucine Found	% L-Leucine Theory	% L-Valine Found	% L-Valine Theory
Leu.leu.leu	44	122–124	50	163–165	HOAc	85	230–231	10.2	10.1	64.2	62.5	<0.1	0
Leu.leu.val	40	129–130	40	166–167	HOAc	60	219–221	10.6	10.4	61.0	65.0	0.2	0
Leu.val.val													
L-L-D + L-D-D	25	148–9.5	50	187–188	HOAc	90	148–150	10.8	10.8	32.5	33.6	20.7	30.3[b]
Leu.val.val													
L-L-D	...	162–163	...	...	...	...	...	...	...	...	...	...	...
Leu.val.leu													
L-L-D + L-D-D	33	150–152	25	177.5-178.5	...	65	205–207	12.0	12.2	34.8	38.1	31.0	34.2[b]
Leu.val.leu													
L-L-D	...	162–164	...	...	...	...	...	...	...	...	...	...	...
Val.val.leu	28	181–182	25	199–200	...	20	270–300[c]	...	...	1.8	0	69.4	71.1
Val.leu.val	55	176–177	75[d]	193–195	...	55	232–233.5	12.7	12.8	35.9	40.0	35.8	35.1
Val.leu.leu	33	170–171	60	182–183	HOAc	45	222–224	9.85	10.4	32.0	32.4	31.0	29.0

[a] Uncorrected. [b] Theory for the pure L-L-D compound. [c] Decomposition. [d] Saponified twice.

directly, without interference from the existing D-amino acid. The limit of the detection is determined by the extent of racemization under the hydrolytic conditions employed. The determination of the D-amino acid which is present due to racemization of the L is made by subtracting the L found from that which should be theoretically present when no racemization occurs.

Maximal assay values for all peptides were obtained after hydrolysis with 4 *N* hydrochloric acid in sealed tubes, after 12–24 hours at 116°, in contrast to the 60 hours at 126° needed for D-valyl-L-valine (4). Increasing the hydrolysis time to 48 hours or longer did not give increased leucine or valine assay values in the peptides val.leu.val, leu.leu.val, or leu.val.leu. The free amino acids D-leucine and D-valine gave 0.7% and about 0.2%, respectively, of the L-forms when hydrolyzed 12 hours, and 3.5% and 1.0% of the L-forms at 60 hours. Approximately 10–20 γ of L-amino acid per ml. in the sample was sufficient for analysis.

Yields of the Z.dipeptide esters of valyl-valine, valyl-leucine, leucyl-valine, and leucyl-leucine were satisfactory. Alkaline saponification of these Z. dipeptide esters also proceeded smoothly and in good yield (Table I).

In the Z.tripeptide series (Table II), yields of the crude Z.tripeptide esters were from 60–80% of crystalline material. However, after several recrystallizations (needed to obtain constant melting point and analytic purity, indicated by amino acid analysis) the yields of pure Z.tripeptide esters were from 25–35%. Saponification proceeded quite slowly compared with the Z.dipeptide esters. After 16 hours, yields of Z.tripeptides from 25–60% were obtained, with most of the remaining Z.tripeptide ester recoverable. There were distinct differences in the yields of the free tripeptides after hydrogenation. N-Terminal leucine peptides were obtained in 60–90% yields; N-terminal valine peptides in 20–55% yields. The N-terminal valine tripeptides which are reported here were obtained optically pure after recrystallization, but this is not conclusive proof of absence of racemization on synthesis (28).

Two of the four protected N-terminal leucyl peptides were obtained in both optical forms. Previously (4), with the phthaloyl blocking group and carbodiimide coupling, the eight optically active tripeptides of L- and D-valine were obtained pure. This probably was due in large part to the use of valine in the N-terminal position, as has been noted above, since Anderson (6) has reported that the carbodiimide procedure also permits racemization to occur.

The data in Table II indicate that no significant racemization occurred on the N-terminal or C-terminal amino acids during coupling, saponification, or hydrogenation. However, leu.val.val was found to be low in L-valine. A second coupling was fractionally crystallized as the Z.tripeptide ester, and yielded both the desired L-L-D peptide ester and the mixture of L-L-D and L-D-D. The Z.L-L-D peptide esters melted some 30° higher than the mixture of L-L-D and L-D-D (Table III).

Rotations, R_f values, and inhibitory powers of the free peptides are given in Table IV. No resolution of the mixtures of L-L-D and L-D-D occurred in the butanol:acetic acid:water system used. Previously (4), we obtained R_f's of 0.70 and 0.73, respectively, for the L-L-D and L-D-D trivalines.

TABLE III.—L-L-D AND L-DL-D Z. TRIPEPTIDE ESTERS OF LEUCINE AND VALINE

Compound	M.P., °C.	% L-Leucine Found	% L-Leucine Theory	% L-Valine Found	% L-Valine Theory	$[\alpha]_D$[a]
Z.leu.val.val.OMe L-L-D	162–163	27.2	27.6	20.9	24.6	−35.1
Z.leu.val.val.OMe L-L-D + L-D-D	131–132	27.1	27.6	13.2	24.6[b]	−22.8
Z.leu.val.leu.OMe L-L-D	162–164	27.6	26.6	24.0	24.0	−27.6
Z.leu.val.leu.OMe L-L-D + L-D-D	126–128	27.5	26.6	12.4	24.0[b]	−10.1

[a] C = 2, $CHCl_3$; T = 25°. [b] Theory for the pure L-L-D compound.

TABLE IV.—PROPERTIES OF L-L-D TRIPEPTIDES OF LEUCINE AND VALINE

Compound	$[\alpha]_D$	R_f[b]	ID_{50},[a] mg./ml. *P. cerevisiae* Parent	ID_{50},[a] mg./ml. *P. cerevisiae* Mutant
Leu.leu.leu.HOAc	+7.4[c]	0.84	0.4	0.3
Leu.leu.val.HOAc	+22.0[c]	0.81[d]	I[e]	0.4
Leu.val.val.HOAc L-L-D + L-D-D	+23.5[g]	0.75	0.2[f]	0.3[f]
Leu.val.leu L-L-D + L-D-D	+22.0[c]	0.78	I[f]	0.2[f]
Val.val.leu		0.75	0.01	0.06
Val.leu.val	+ 6.6[c]	0.75[d]	I	I
Val.leu.leu.HOAc		0.75	I	I
Val.val.val	−37.0[c,g] −37.3[i]		0.03 ± 0.03	0.03 ± 0.01[h]

[a] Concentration at which growth is reduced 50%, maximum test level 0.25 or 0.50 mg./ml. [b] Solvent butanol:water:acetic acid 10:8:1 upper phase, compounds applied as hydrochlorides. [c] C = 2, 1 *N* HCl; T = 25°. [d] Very small second spot at R_f 0.34. [e] I = Inhibition occurred less than 50%. [f] The L-D-D tripeptide of valine was inert (2). [g] Reported −41.8 (4). [h] This compound also inhibited *L. plantarum*, the other compounds stimulated this growth of *L. plantarum* under the test conditions. [i] C = 2, H_2O.

SUMMARY

Seven possible tripeptides of leucine and valine of the L-L-D configuration were synthesized.

Their optical purity was determined bacterimetrically.

These peptides were not effective as antibacterial agents compared with L-valyl-L-valyl-D-valine.

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Stability of Pyridine-2-aldoxime Methiodide II

Kinetics of Deterioration in Dilute Aqueous Solutions

By ROBERT I. ELLIN, JAMES S. CARLESE, and ALBERT A. KONDRITZER

The decomposition of 2-PAM was studied at pH values 0.5 to 13 and at temperatures ranging from 37 to 87°. At constant pH and temperature, the observed rate of deterioration of 2-PAM was first order with respect to drug concentration. Below pH approximately 4, hydrogen ion catalysis of the acid form of oxime occurs; above pH 4, the reaction is either an hydroxyl ion catalysis of the acid form of oxime or is noncatalytic in which there is a direct attack on the oximate species. Two mechanisms have been postulated for the breakdown of 2-PAM. Specific velocity constants, energies of activation, and frequency factors have been determined for both mechanisms. The pH of maximum stability has been determined from initial rate constants over the entire pH range. General equations relating the half-life of 2-PAM solutions to temperature and pH have been derived.

DURING the past few years a group of new drugs, of which the best known is pyridine-2-aldoxime methiodide (2-PAM, 2-formyl-1-methylpyridinium iodide oxime), has been introduced which is capable of reversing neuromuscular block caused by organophosphates and may be of clinical value in the treatment of intoxication by these compounds (1–6). Aqueous solutions of 2-PAM, contained in injector items, may be used for purposes of self-aid and first-aid. Therefore, knowledge of the stability in water is essential. The object of this study was to establish a basis for predicting the rate of decomposition of 2-PAM at various hydrogen and hydroxyl ion concentrations and temperatures.

The hydrolysis and pyrolysis of acylated oximes have been the subject of many investigations (7–9). The hydrolysis of free oximes in acid solution has been well investigated (10). However, relatively few references are available on the alkaline decomposition of the free oxime. For the most part, these reports were not concerned with the kinetics of the process or predictions based on such data. Benger and Brady (11) presented various mechanisms for the alkaline hydrolysis of acyl benzaldoximes. Two of the mechanisms described were attack by hydroxyl ions: (*a*) at the methine hydrogen atom resulting in formation of the corresponding nitrile and (*b*) at the carbonyl carbon resulting in the formation of free oxime. In general, aldoximes on reaction with alkali do not give rise to the corresponding nitrile because the high electron density on the oxygen prevents attack by hydroxyl ions at the methine hydrogen atom. Replacement of the hydrogen of the hydroxyl group of the oxime by an acyl group establishes a relative positive charge at the oximino oxygen, thereby reducing the electron density sufficiently to permit hydroxyl ion attack at the methine hydrogen atom (11, 12). This mechanism is considered to involve removal of the methine hydrogen atom as a proton accompanied or followed by release of the acylate anion (11, 13). On the other hand, the free aldoxime, *o*-nitrobenzaldoxime, on heating with aqueous sodium hydroxide gives rise to the corresponding nitrile. Here the negative inductive effect of the nitro group facilitates the removal of a proton at the methine hydrogen atom (14, 15).

The decomposition of 2-PAM takes place by two mechanisms. Below pH approximately 4, 2-PAM is hydrolyzed to pyridine-2-carboxaldehyde methiodide and hydroxylamine (reaction 2, Fig. 1). Above pH 4, 2-PAM is dehydrated to 2-cyanopyridine methiodide, which is converted to 2-hydroxypyridine methiodide, which rearranges to N-methyl-alpha-pyridone (16) (reaction 1, Fig. 1). The latter reaction may follow a mechanism similar to the one mentioned above. The nitrogen on the pyridine ring, being strongly electrophylic, would have an effect similar to that of a nitro group and thereby facilitate hydroxyl ion attack on the methine hydrogen atom.

The kinetics of the decomposition of 2-PAM was studied over the pH range 0.5 to 13 and at the temperatures 37, 57, and 87°. Kinetic constants, determined at the various temperatures, were used to calculate the activation energy for each of the two mechanisms. From these values an equation was derived for predicting the decomposition of 2-PAM at any particular temperature and pH. By taking into account the effects of both hydroxyl and hydrogen ions, the pH of minimum decomposition at various tem-

Received January 14, 1961, from the Physiology Division, Directorate of Medical Research, U. S. Army Chemical Research and Development Laboratories, Army Chemical Center, Md.
Accepted for publication March 28, 1961.
Part I was published in *J. Am. Chem. Soc.*, **80**, 6588(1958).

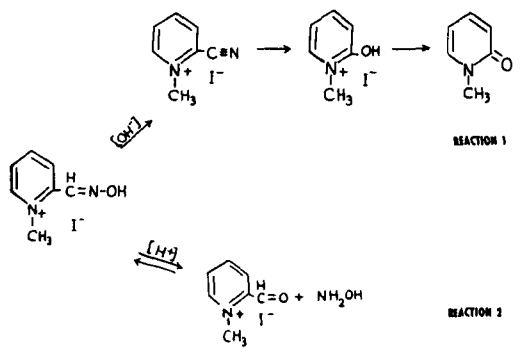


Fig. 1.—Reactions involved in the decomposition of 2-PAM.

peratures and the decomposition rates at these minimum pH values were calculated. By using initial rate constants, the half-life of 2-PAM solutions have been calculated for various pH values and temperatures.

EXPERIMENTAL

The method of analysis consisted of making a series of ultraviolet measurements of 2-PAM solutions undergoing decomposition. (2-PAM, purchased from Aldrich Chemical Co., Milwaukee, Wis., was twice recrystallized from ethyl alcohol and has a melting point of 228–230°.) Ultraviolet absorption spectra of 2-PAM and its acid and base decomposition products were determined in acid and alkaline solutions and indicated that 2-PAM can be determined quantitatively in the presence of its decomposition products at 335 mμ in 0.1 *N* sodium hydroxide (17).

The hydrogen and hydroxyl ion concentrations of the system were maintained by using buffer solutions or an excess of acid and base. The pH change during the experimental period was limited to 0.1 pH unit. Buffer systems used in the study are listed in Table I. From pH 0.4 to 1.2, the pH was controlled by an excess of perchloric acid; at pH 13, by an excess of sodium hydroxide. The pH of each buffer was determined at the temperatures at which the experiments were carried out.

Table I.—List of Buffers Employed and Composition of Each

pH	Composition	Dilution
2	5.0 ml. 0.2 *M* KCl + 10.6 ml. 0.2 *N* HCl	to 200 ml.
3	3 Gm. glacial acetic acid	to 500 ml.
4	16 ml. 0.1 *N* acetic acid + 4 ml. of 0.1 *N* sodium acetate	as is
5	6 ml. of 0.1 *N* acetic acid + 14 ml. of 0.1 *M* sodium acetate	as is
6	11.6 Gm. KH_2PO_4 + 2.15 Gm. Na_2HPO_4	to 500 ml.
7	4.55 Gm. KH_2PO_4 + 9.45 Gm. Na_2HPO_4	to 500 ml.
8	5.9 Gm. boric acid + 4.55 Gm. borax	to 500 ml.
10	3.25 Gm. $NaHCO_3$ + 6.6 Gm. Na_2CO_3	to 500 ml.
12	12.3 Gm. Na_3PO_4	to 500 ml.

Buffer solutions were placed in a thermostatically controlled bath, and allowed to reach the temperature of the bath. A volume of standard solution, containing 25 mg./ml. of 2-PAM, was then added to give the reaction mixture a concentration of 1 mg./ml. The decomposition of 2-PAM was determined by periodically removing 1-ml. samples, diluting to 100 ml. with 0.1 *N* sodium hydroxide, and following the disappearance of the oxime grouping with a Beckman DU quartz spectrophotometer at 335 mμ.

RESULTS AND DISCUSSION

The observed degradation of 2-PAM under various conditions, examples of which are given in Figs. 2–4, conform to the classical first-order equation, $\ln c = kt +$ constant, where k is the first-order rate constant at a constant pH. 2-PAM as the free oxime represents the protonated form of the oxime and will be designated (OxH). The protonated form is positively charged as the nitrogen of the pyridine ring of 2-PAM is in the charged form. The basic or oximate species of 2-PAM may be considered to be electrically neutral as the negative charge on the oximino oxygen internally neutralizes the positive charge on the pyridine ring nitrogen. The oximate species will be designated (Ox).

From the pH profile shown in Fig. 5 we can assume that the hydrolysis of 2-PAM at pH values 0–4 is due to the hydrogen ion catalysis of the protonated species. At pH values 4 to 8, the mech-

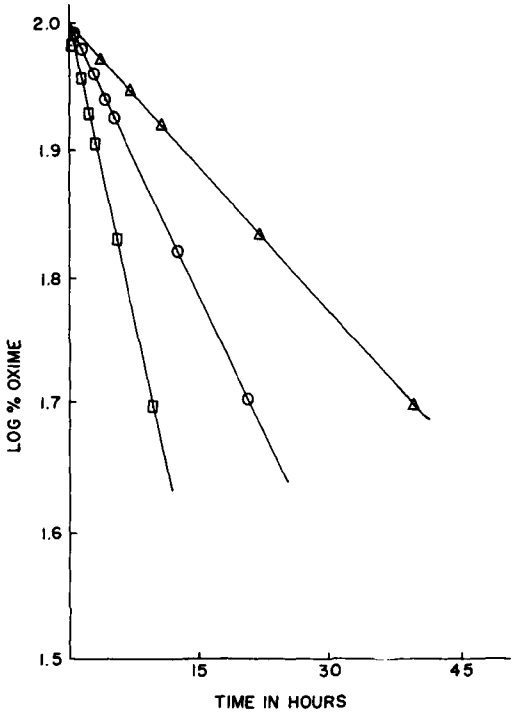


Fig. 2.—Typical first-order rate plots for the acid catalyzed decomposition of 2-PAM at 57°. △, 0.096 *N*; ○, 0.191 *N*; □, 0.383 *N* perchloric acid.

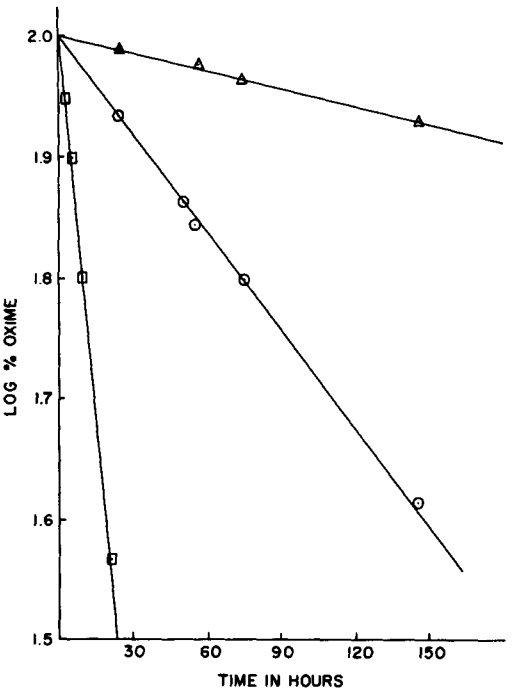


Fig. 3.—Typical first-order rate plots for the decomposition of 2-PAM at pH values 5 to 7 at 57°. △, pH 5; ○, pH 6; □, pH 7.

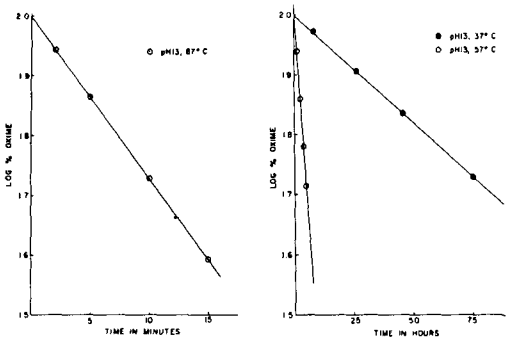


Fig. 4.—Typical first-order rate plots for the decomposition of 2-PAM at pH 13 at various temperatures.

anism appears to involve hydroxyl ion catalysis of the oxime species of 2-PAM. However, a pH independent region is observed at the pH values approximately 8–13. Thus at pH values 4 to 13, equations can be derived to show the mechanism of degradation involves either hydroxyl ion catalysis of the oxime species or a direct attack on the oximate species of 2-PAM, in which there is no catalysis.

The symbol k_{H^+} represents the catalytic rate constant or catalytic coefficient for the hydrogen ion catalysis of the protonated species, k_{OH^-} represents the catalytic constant for the hydroxyl ion catalysis of the protonated species, k_{Ox} represents the degradation constant acting directly on the oximate species of 2-PAM, a is the total drug concentration, and Ka

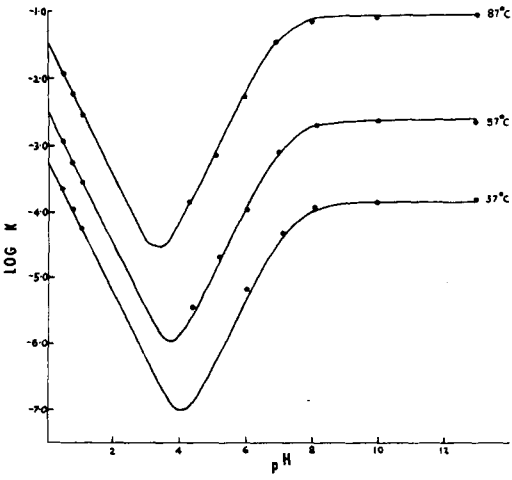


Fig. 5.—The pH profile of the logarithm of initial first-order rate constants for the breakdown of 2-PAM at 37, 57, and 87°. The curves represent values calculated from the equation derived from experimental data. The illustrated points represent experimental values.

and Kw are the acid and water dissociation constants, respectively. Then

$$\frac{d \ln a}{dt} = k_{H^+}(H^+)\frac{(H^+)}{Ka + (H^+)} + \frac{k_{OH^-}\, kw}{Ka + (H^+)} \qquad \text{(Eq. 1)}$$

or

$$\frac{d \ln a}{dt} = k_{H^+}(H^+)\frac{(H^+)}{Ka + (H^+)} + \frac{k_{Ox}\, Ka}{Ka + (H^+)} \qquad \text{(Eq. 2)}$$

The right sides of Eqs. 1 and 2 are constant at any given pH. The overall rate of degradation is first order with respect to oxime concentration and $(d \ln a/dt)\text{pH} = k$, the overall pseudo first-order rate constant at a constant pH.

Whenever the first term of Eqs. 1 and 2 is important, that is, the value of the hydrogen ion concentration is much larger than the value of Ka, the oxime exists in the protonated form. Therefore, at any pH the equations reduce, respectively, to

$$k = k_{H^+}(H^+) + \frac{k_{OH^-}\, kw}{Ka + (H^+)} \qquad \text{(Eq. 3)}$$

and

$$k = k_{H^+}(H^+) + \frac{k_{Ox}\, Ka}{Ka + (H^+)} \qquad \text{(Eq. 4)}$$

Hence, k_{OH^-} will be related to k_{Ox} at any given pH and temperature by the statement

$$k_{Ox} = k_{OH^-}\, Kb \qquad \text{(Eq. 5)}$$

where Kb is the basic dissociation constant of 2-PAM.

At relatively high pH values, approximately 10 to 14, Eq. 3 reduces to $k = k_{OH^-}$ Kb and Eq. 4 reduces to $k = k_{Ox}$. At relatively low pH values below 3, Eqs. 3 and 4 reduce to $k = (k_{OH^-}\, Kw)/Ka + (H^+)$ and Eq. 4 reduces to $k = k_{Ox}Ka/Ka + (H^+)$.

TABLE II.—OBSERVED RATE CONSTANTS (k in min.$^{-1}$) FOR THE SPECTROPHOTOMETRICALLY DETERMINED DEGRADATION OF 2-PAM

37°		57°		87°	
pH	k, min.$^{-1}$	pH	k, min.$^{-1}$	pH	k, min.$^{-1}$
13	1.54×10^{-4}	13	2.17×10^{-3}	13	8.77×10^{-2}
9.96	1.39×10^{-4}	9.98	2.15×10^{-3}	9.81	7.81×10^{-2}
8.05	1.15×10^{-4}	8.14	2.01×10^{-3}	8.01	6.62×10^{-2}
7.10	4.53×10^{-5}	7.02	8.03×10^{-4}	6.91	3.30×10^{-2}
5.88	6.06×10^{-6}	6.06	1.04×10^{-4}	5.95	5.23×10^{-3}
		5.21	1.95×10^{-5}	5.10	6.96×10^{-4}
		4.32	3.65×10^{-6}	4.30	1.36×10^{-4}
1.02	5.60×10^{-5}	1.02	2.89×10^{-4}	1.02	2.75×10^{-3}
0.72	1.15×10^{-4}	0.72	5.36×10^{-4}	0.72	5.62×10^{-3}
0.42	2.25×10^{-4}	0.42	1.15×10^{-3}	0.42	1.15×10^{-2}

TABLE III.—PREDICTED STABILITY OF 2-PAM AT pH OF MINIMUM HYDROLYSIS

t, °C.	pH min.	$t^{1}/_{2}$, yr.
0	4.93	3,000
10	4.68	800
20	4.44	160
25	4.36	80
30	4.23	38
37	4.07	13.5
40	4.02	9.5
50	3.85	2.5
57	3.74	1.15
60	3.69	0.75
70	3.54	0.25
80	3.40	0.1
87	3.32	0.05
100	3.20	5 days

Between the regions of high and low pH the reaction velocity will pass through a minimum at a hydrogen ion concentration which is found by differentiating k with respect to (H^+) in Eqs. 3 or 4, and equating to zero. For Eq 3

$$k\,H^+ - \frac{k_{OH^-}\,Kw}{(Ka + H^+)^2} = 0 \qquad \text{(Eq. 6)}$$

At the observed pH of minimum decomposition, shown to be about pH 4, Ka is small compared to H^+ and can be dropped from the equation. Then

$$(H^+_M)^2 = \frac{k_{OH^-}\,Kw}{k_{H^+}} \qquad \text{(Eq. 7)}$$

For Eq. 4

$$(H^+_M)^2 = \frac{k_{Ox}\,Ka}{k_{H^+}} \qquad \text{(Eq. 8)}$$

The value k_M, the velocity constant at $(H^+)_M$, is obtained by substituting in Eqs. 3 and 4 the corresponding values of $k_{OH^-}\,Kw$ in Eq. 7 and $k_{Ox}\,Ka$ in Eq. 8. In either case

$$k_M = 2\,k_{H^+} + (H^+)_M \qquad \text{(Eq. 9)}$$

Temperature Dependency of the Reactions.—Velocity constants were determined at 37, 57, and and 87° in acid solutions which were approximately 0.1, 0.2, and 0.4 N with respect to hydrogen ion concentration. These measurements, which represent a type 2 reaction, were used to calculate the kinetic constants at 1 N hydrogen ion concentration for each of the temperatures used. See Table II. The values obtained for the latter constant are: k_H at 37° = 5.84×10^{-4} min.$^{-1}$; k_{H^+} at 57° = 2.97×10^{-3} min.$^{-1}$; and k_{H^+} at 87° = 2.98×10^{-2} min.$^{-1}$. In Fig. 6, the velocity constants are plotted against the reciprocal of the absolute temperatures. The straight line relationship indicates that the mechanism responsible for the oxime hydrolysis is not altered with change in temperature. The relationship between temperature and velocity constants is shown by the Arrhenius equation

$$k_{H^+} = Ze^{-Ea/RT} \qquad \text{(Eq. 10)}$$

On calculating the slope and setting it equal to $-Ea/2.303$ RT, the energy of activation, Ea, was found to be approximately 17,400 calories per mole. The frequency factor in the Arrhenius equation, Z, was found by substituting the conditions at the three experimental temperatures. The average Z was found to be 1.024×10^9 min.$^{-1}$.

It is evident from Fig. 5 that above pH 4, the

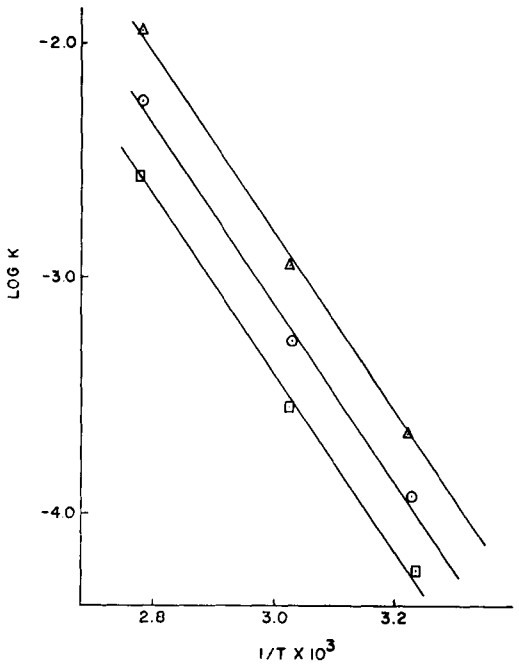


Fig. 6.—Temperature dependency of reaction 1 the hydrolytic reaction of 2-PAM, catalyzed by hydrogen ions, logarithm of k min.$^{-1}$ plotted against the reciprocal of the absolute temperature. △, 0.383 N; ○, 0.191 N; □, 0.096 N perchloric acid.

overall reaction is essentially that of reaction 1. As the exact mechanism by which reaction 1 progresses has not been established, two energy of activation constants were determined, one for the potentially base catalyzed and one for the potentially uncatalyzed reaction. The logarithms of velocity constants for each mechanism, determined at pH values 5 to 13, were plotted against the reciprocals of the absolute temperature. Representative results, shown in Fig. 7, again indicate that neither mechanism for the dehydration reaction is altered with change in temperature. The average slope of the uncatalyzed reaction was found to be 6,200, the activation energy, *Ea*, being 28,500 calories per mole. The energy of activation of the base catalyzed reaction was found to be 17,000 calories per mole. By

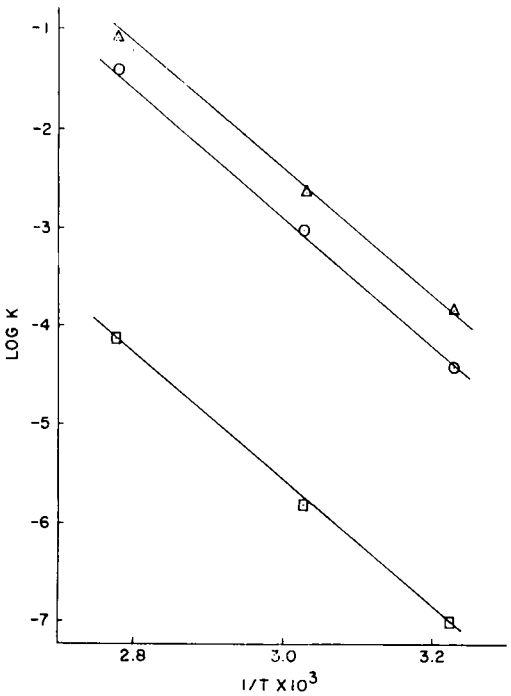


Fig. 7.—Temperature dependency of reaction 2, the dehydration reaction of 2-PAM at pH values 4, 7, and 10. □, pH 4; ○, pH 7; △, pH 10.

substituting the value of k_{Ox} and of k_{OH^-} at various experimental temperatures in the Arrhenius equation, the frequency factors come out to be 1.797×10^{16} min.$^{-1}$ and 3.144×10^{14} min.$^{-1}$, respectively.

The overall equation for the breakdown of 2-PAM at various pH values is either

$$k = 1.02 \times 10^{9}\, e^{-17,400/RT}(H^+) + 1.8 \times 10^{16}\, e^{-28,500/RT} \frac{Ka}{Ka + (H^+)} \quad \text{(Eq. 11)}$$

or

$$k = 1.02 \times 10^{9}\, e^{-17,400/RT}(H^+) + 3.1 \times 10^{14}\, e^{-17,000/RT} \frac{k_{OH^-}\, Kw}{Ka + (H^+)} \quad \text{(Eq. 12)}$$

pH of Minimum Hydrolysis.—The hydrogen ion concentration at which minimum decomposition occurs is found at the point where the first term of the right-hand side of Eq. 11 or 12 equals the second term. On substituting into Eq. 8 the values of k_{H^+} and k_{Ox}, the equation for the pH of minimum decomposition pH_M, may be expressed as

$$pH_M = {}^1/_2 \left(pKa - 7.246 + \frac{2436}{T}\right) \quad \text{(Eq. 13)}$$

The pH of minimum decomposition changes inversely with changes in temperature, ranging from 4.68 at 0° to 3.32 at 87° (Table III). The pKa values at various temperatures may be estimated by substituting the corresponding experimental values into Eq. 4. As the pKa values for 2-PAM were found experimentally to be between 7 and 8, the $k_{H^+}(H^+)$ becomes small and may be eliminated. Thus

$$Ka = \frac{k_{H^+}}{k_{Ox} - k} \quad \text{(Eq. 14)}$$

Using Eq. 11 or 12 the half-lives of 2-PAM can be calculated at any pH value and temperature. Predicted stability data over the region of pH 3 to 8 at temperatures from 10 to 87° are given in Table IV. The half-lives near the pH of minimum decomposition were, in most instances, extrapolated from initial rate constants as the half-lives are so long that experimental determinations were not practical.

Equilibrium.—In addition, we encountered in the acid region of pH 1 to 3, various conditions of equilibrium between 2-PAM and its hydrolysis products, pyridine - 2 - carboxaldehyde methiodide

TABLE IV.—PREDICTED STABILITY OF 2-PAM IN AQUEOUS SOLUTION, $t^1/_2$ (HALF-LIFE)

Temp., °C.	pH[a] 8	7	6	5	4	3
10	1.6 y	8.7 y	80 y	640 y	330 y	34 y
20	85 d	1 y	9 y	86 y	106 y	12 y
25	35 d	145 d	3.5 y	33 y	60 y	7 y
30	12 d	45 d	1 y	10 y	31 y	4.5 y
37	4.6 d	15 d	115 d	3 y	13.5 y	2.5 y
40	2.9 d	9.5 d	74 d	2 y	9 y	1.8 y
50	16 h	46 h	14.5 d	135 d	2.5 y	0.8 y
57	6 h	16 h	4.5 d	45 d	350 d	154 d
60	4 h	9.5 d	2.5 d	26 d	213 d	120 d
70	1.1 h	2.5 h	17 h	7 d	60 d	50 d
80	20 m	40 m	4.5 h	40 h	16 d	23 d
87	9 m	20 m	1.8 h	18 h	7 d	14 d

[a] y = years, d = days, h = hours, m = minutes.

(PCAM) and hydroxylamine. An equilibrium constant, $k_{eq.}$, can be determined at any pH value. The equilibrium constant for reaction 2 is

$$k_{eq.} = \frac{(PCAM)(NH_2OH)}{(2\text{-}PAM)} \qquad \text{(Eq. 15)}$$

The basic dissociation constant for hydroxylamine may be arranged as

$$\frac{(NH_2OH)}{(NH_3OH)^+} = \frac{(OH^-)}{Kb} = \frac{Kw}{Kb\,(H^+)} \qquad \text{(Eq. 16)}$$

Substituting Eq. 16 into Eq. 15 and since the concentration of $(NH_3OH)^+$ is equal to the concentration (PCAM)

$$k_{eq.} = \frac{Kw\,(PCAM)^2}{Kb(H^+)\,(2\text{-}PAM)} \qquad \text{(Eq. 17)}$$

The value of $k_{eq.}$ for the reactions in our study at 87° was approximately 20.

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Synthesis of Some N-Aralkyl-N-methylaminoethyl Carbanilates

By ROGER W. BARNES and WILLIAM J. ROST

In an attempt to correlate the basicity of local anesthetics with their activity, 21 substituted carbanilates of N-benzyl-N-methylaminoethanol, N-phenethyl-N-methylaminoethanol, and N-phenpropyl-N-methylaminoethanol were prepared. Substitutions on the carbanilic acid portion of the molecule were designed to study possible resonance effects. The compounds showed topical local anesthetic properties, but infiltration anesthetic properties were almost absent. No correlation between basicity and local anesthetic properties could be made. The relatively high acidity of the hydrochloride salts tested might account for the irritation commonly found.

CARBANILIC ACID esters of aminoalcohols have been reported to have marked local anesthetic activity (1, 2). Carbanilates with alkyl, chloro, amino, and alkoxy substitutions have been shown to possess a fair to high degree of local anesthetic activity (3–11). The position of the substitutions on the phenyl group have favorably affected the activity of the compounds in some cases (4, 6, 9). Sekera and Vrba have concluded that the more stable carbamates had a greater activity (6).

Aralkyl substitution on the nitrogen of aminoalkyl esters has given compounds with marked local anesthetic action. A 2% solution of γ-(2-benzylpiperidino)propyl benzoate or γ-(2-phenethylpiperidino)propyl benzoate gave a corneal anesthesia lasting 5 to 6 days (12). β-(N-Methyl-N-phenethylamino)ethyl carbanilate administered intracutaneously to a guinea pig in 0.5% concentration, gave an anesthetic effect lasting more than 24 hours. The local anesthetic effect of this compound lasted 84 minutes when used on the rabbit cornea (13).

Because the aromatic substitutions gave a marked increase in the activity of dialkylaminoalkyl carbanilates and because N-aralkylation of the aminoalkyl benzoates, *p*-aminobenzoates, and the carbanilate proved effective, it was thought to be of interest to investigate the N-aralkylation of aminoalkyl esters of substituted carbanilic acids. Tolstoouhov has postulated that the local anesthetic effect of a molecule is related to the basicity of the amine nitrogen it contains (14). The N-aralkyl groups chosen for investigation were benzyl, phenethyl, and phenpropyl. The N substitutions might then vary the basic charac-

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ter of the amine and therefore vary the activity of the compounds as local anesthetics. Carbanilates, methylcarbanilates, chlorocarbanilates, methoxycarbanilates, and nitrocarbanilates of the N-phenalkyl-N-methylaminoethanols were prepared. The nitrocarbanilates could then be reduced to the amino derivatives after the initial reaction. These substituted carbanilates were chosen to vary the resonance effects of the carbamic acid esters. *Meta-* and *para-*substitutions were chosen to contrast the different resonance effects. *Ortho-* substituted carbanilates were not studied because of the possible steric hindrance.

The purpose of this paper is to report the synthesis of 21 compounds, report their basicities, and to give a preliminary evaluation of their local anesthetic activities.

EXPERIMENTAL

N - Phenalkyl - N - methylaminoethanols.—The proper phenalkyl halide was condensed with two equivalents of N-methylaminoethanol in the absence of a solvent. The exothermic reactions were first cooled in an ice bath and then allowed to stand at room temperature for varying lengths of time. After treatment with aqueous sodium hydroxide, the reaction mixture was extracted with ether. The ether was evaporated, and the aminoalcohol was distilled under reduced pressure. The products are described in Table I.

Carbanilates.—A solution of 0.066 *M* of the appropriate isocyanate dissolved in 30 ml. of dry toluene was slowly added to 0.06 *M* of the N-phenalkyl-N-methylaminoethanol. After the initial heat of reaction had subsided, the mixture was refluxed for 3 hours and allowed to stand at room temperature for 2 days. At the end of this period, the urea which had formed was filtered from the solution. Anhydrous hydrogen chloride was passed into the filtrate to obtain an oil which changed on standing to the solid hydrochloride salt. The crude product was recrystallized from acetone to which a trace of ethanol had been added.

Reduction of Nitrocarbanilates.—The nitrocarbanilates on reduction with hydrogen under low pressure with platinum oxide yielded an oil which solidified on standing. Attempts at recrystallization were unsuccessful.

The carbanilates prepared are listed in Table II.

Comparison of Basicities of Derivatives.—The basicities of the various compounds were compared using the method of Tolstoouhov (14). The pKb of each compound was also determined. A solution of the amine hydrochloride was dissolved in water. The salt was half neutralized by the addition of 0.01 *N* sodium hydroxide. Because of the insolubility of the free base, alcohol was added to facilitate solution. The pH of the resultant solution was determined, and the pKb calculated. The results of the above determinations are reported in Table III.

TABLE I.—AMINOALCOHOLS

Compound	B.p., °C.	% Yield
$C_6H_5—CH_2—N(CH_3)—CH_2CH_2OH$	124–129/8 mm. Hg	83
$C_6H_5—(CH_2)_2—N(CH_3)—CH_2CH_2OH$	143–145/10 mm. Hg	51
$C_6H_5—(CH_2)_3—N(CH_3)—CH_2CH_2OH$	173–178/20 mm. Hg	66

TABLE II.—N-PHENALKYL-N-METHYLAMINOETHYL CARBANILATES
$R—C_6H_4—HN—CO—O—CH_2CH_2N(CH_3)R'$

R	R'	M.P., °C.	Yield, %	Analysis: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
H	$C_6H_5CH_2$—	167–168	58.2	63.64	63.28	6.60	6.23
m CH_3—	$C_6H_5CH_2$—	167–168	55.7	64.56	63.86	6.92	6.33
p CH_3—	$C_6H_5CH_2$—	210–212	56.9	64.56	64.58	6.92	6.36
m Cl—	$C_6H_5CH_2$—	165–166	72.7	57.47	56.50	5.67	5.34
p Cl—	$C_6H_5CH_2$—	209–211	60.0	57.47	57.67	5.67	5.59
p CH_3O—	$C_6H_5CH_2$—	169–170	42.7	61.62	61.72	6.61	6.73
m O_2N—	$C_6H_5CH_2$—	118–120	36.4	...	...	...	...
p O_2N—	$C_6H_5CH_2$—	212–214	42.7	...	...	...	...
H	$C_6H_5(CH_2)_2$—	186–188	69.0	64.56	64.67	6.92	6.56
p CH_3—	$C_6H_5(CH_2)_2$—	208–210	77.5	65.41	65.53	7.22	7.05
p Cl—	$C_6H_5(CH_2)_2$—	210–212	95.0	58.54	58.94	6.01	5.84
p CH_3O—	$C_6H_5(CH_2)_2$—	192–193	82.5	62.54	62.47	6.91	6.67
p O_2N—	$C_6H_5(CH_2)_2$—	97	78.7	62.96	62.62	6.17	6.28
H	$C_6H_5(CH_2)_3$—	165–166	65.4	65.41	65.30	7.22	7.00
m CH_3—	$C_6H_5(CH_2)_3$—	139–140	59.1	66.19	65.26	7.50	7.28
p CH_3—	$C_6H_5(CH_2)_3$—	165–166	78.3	66.19	66.37	7.50	6.93
m Cl—	$C_6H_5(CH_2)_3$—	185–186	69.4	59.53	58.85	6.31	6.72
p Cl—	$C_6H_5(CH_2)_3$—	165–166	51.1	59.53	59.49	6.31	6.38
p CH_3O—	$C_6H_5(CH_2)_3$—	135–137	58.1	63.40	63.64	6.92	6.99
m O_2N—	$C_6H_5(CH_2)_3$—	161–162	39.6	...	...	...	...
p O_2N—	$C_6H_5(CH_2)_3$—	165–167	58.8	...	...	...	...

TABLE III.—BASICITY OF CARBANILATES

Substitution	pH at Which Solution Becomes Cloudy[a]	pKb
Substituted Carbanilates of N-Benzyl-N-methylaminoethanol		
H	6.15	7.40
m-Methyl	5.59	7.42
p-Methyl	5.59	7.47
m-Chloro	5.19	7.58
p-Methoxy	6.09	7.37
Substituted Carbanilates of N-Phenethyl-N-methylaminoethanol		
H	6.19	7.10
p-Methyl	5.77	7.02
p-Chloro	5.38	7.05
p-Methoxy	6.12	7.14
Substituted Carbanilates of N-Phenpropyl-N-methylaminoethanol		
H	5.59	6.82
m-Methyl	5.75	6.88
m-Chloro	5.15	6.92
p-Chloro	5.35	7.01
p-Methoxy	6.11	7.11

[a] Done according to method of Tolstoouhov (14).

PHARMACOLOGY

The local anesthetic activity of the compounds was determined on the mouse cornea. With two exceptions, the compounds were active in concentrations of 0.5% or less. N-Benzyl-N-methylaminoethyl *p*-methylcarbanilate and N-benzyl-N-methylaminoethyl *m*-methylcarbanilate were active in concentrations as low as 0.05%. Irritation was commonly found. Only the N-benzyl-N-methylaminoethyl carbanilate, *m*-methylcarbanilate, and *p*-methylcarbanilate showed local anesthetic activity when injected intradermally into the guinea pig back. Irritation was commonly found when this method of testing was used.

The N-benzyl-N-methylaminoethyl carbanilate, *m*-methylcarbanilate, and *p*-methylcarbanilate were tested for any possible action they might have on the rat intestine. A decrease in motility was observed with the most marked decrease being with the N-benzyl-N-methylaminoethyl *p*-methylcarbanilate.

The minimum lethal doses of these compounds was of the order of 200 mg./Kg.

DISCUSSION

Tolstoouhov has postulated that compounds whose basicity fell within a certain range would be local anesthetics (14). Local anesthetic salts which start to separate from solution as the free base at pH 5.93–6.36 were claimed to have the highest order of activity. Compounds possessing a basicity outside of this optimum range were shown to have a gradually diminishing activity. The above postulation was not found to hold in this series of carbanilates. The best local anesthetic activity was found in the N-benzyl-N-methylaminoethyl carbanilates. Although compounds in the other series possessed a basicity which fell within the optimum range suggested by Tolstoouhov, they showed no intradermal local anesthetic activity. Almost all of the compounds, however, did show a topical activity. No correlation between basicity and local anesthetic activity was possible.

The pKb of the compounds indicated that they were relatively weak bases. The acidity of the hydrochloride salts tested could then explain the irritation commonly found in this series. A similar explanation might be given for the irritant compounds reported by Sievers and McIntyre (13).

The electronic influences of the aromatic substitutions on the carbanilic acid portion of the molecules seemed to have no effect on the local anesthetic activity.

SUMMARY

The syntheses and properties of 21 N-aralkyl-N-methylaminoethyl carbanilates were described.

The compounds tested possessed a topical local anesthetic effect in concentrations of 0.5% or less. Infiltration anesthetic properties were almost absent.

The relative basicities of the compounds were determined, but no correlation with local anesthetic activity could be made. The relatively high acidity of the hydrochloride salts tested might account for the irritation commonly found.

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Coumarins I

Isolation, Purification, and Structure Determination of Pteryxin and Suksdorfin

By ROBERT E. WILLETTE† and TAITO O. SOINE

Pteryxin and suksdorfin were isolated from petroleum ether extracts of *Pteryxia terebinthina* (Hook.) Coulter and Rose var. *terebinthina* and *Lomatium suksdorfii* (Wats.) Coulter and Rose, respectively. A suitable chromatographic procedure was developed for their purification. Upon hydrolysis, these coumarins yielded the same isomeric alcohols obtained from visnadin and samidin. In addition, an alcohol and its acetate which had not been reported previously were obtained and assigned the structures (+)-*cis*-ethylkhellactone and (−)-*cis*-acetylethylkhellactone. Pteryxin, during the hydrolysis, yielded angelic and acetic acids. Quantitative hydrolytic studies, optical rotations, isolation of the partially hydrolyzed coumarins as acetates, and ultraviolet and infrared absorption studies led to the establishment of the following structure for pteryxin: 3′-acetyl-4′-angeloyl-(+)-*cis*-khellactone; and for suksdorfin: 3′-acetyl-4′-isovaleryl-(+)-*cis*-khellactone. The probable conformation of these substances is discussed.

THE PRESENT investigations were stimulated by the recent interest in the isolation and characterization of various naturally occurring chromones and coumarins from *Umbelliferae* possessing certain and distinct pharmacological activities. The chromone khellin, which is obtained from the umbellifer *Ammi visnaga*, has had some clinical success (1). From the strongly vasodilatory oil which remains after the isolation of khellin, Smith and co-workers reported the isolation and structural determinations of the coumarins visnadin, samidin, and dihydrosamidin (2). Visnadin has undergone clinical studies under the registered name Provismine (3). The absolute configurations of these latter substances were established by Schmid and co-workers (4).

In an extensive pharmacognostical investigation of *Pteryxia terebinthina* (Hook.) Coulter and Rose var. *terebinthina*, Call (5) isolated a low-melting solid, which he named pteryxin, and which exhibited antispasmodic activity (6). Preliminary characterization studies by Pettinato (7) indicated pteryxin to be a coumarin containing two alkali-consuming groups in addition to the expected lactone grouping.

Call (8) isolated another crystalline substance from the fruit of *Lomatium suksdorfii* (Wats.) Coulter and Rose which he named suksdorfin A. He also showed that it possessed antispasmodic properties. A preliminary phytochemical study (9) and an analysis of the volatile oil content (10) of this plant have been reported.

The investigation reported here concerns the isolation, purification, and determination of structure of pteryxin and suksdorfin.

Detailed pharmacological studies will be reported at a later date.

EXPERIMENTAL

Melting points were determined in capillary tubes in an oil bath with a thermometer that read accurately for a set of standard samples. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Perkin and Elmer spectrophotometer, model 137. Ultraviolet spectra were determined in 95% ethanol on Cary recording spectrophotometers, models 11 and 14.

Material.—Dried roots of *Pteryxia terebinthina* (Hook.) Coulter and Rose var. *terebinthina*, collected at an elevation of 400 ft. near Rufus, Oreg., and dried fruits of *Lomatium suksdorfii* (Wats.) Coulter and Rose, collected at an elevation of 1,200 ft. near Klickitat, Wash., were ground in a Jacobsen laboratory mill to an approximately No. 20 powder in preparation for extraction.[1]

Isolation of Pteryxin (I) from *P. terebinthina*.—The ground root (1 Kg.) was washed with two 2-L. portions of ice-cold petroleum ether (b.p. 30–60°), which were discarded. The washed root was boiled with three 2-L. portions of Skellysolve B (b.p. 65–70°); the menstruum after each extraction was filtered into a large beaker. Upon cooling, a resinous, oily deposit separated, from which the clear liquor was decanted. The liquor became cloudy on pouring and, after standing overnight, was decanted

Received March 31, 1961, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis.

Accepted for publication May 4, 1961.

Abstracted in part from a dissertation presented to the Graduate School, University of Minnesota, by Robert E. Willette in partial fulfillment of the requirements for the degree of Doctor of Philosophy, July 1960.

Presented to the Scientific Section, A.PH.A., Washington, D. C., meeting, August 1960.

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The authors are indebted to The Upjohn Co., Kalamazoo, Mich., for making available the instruments used for much of the spectroscopic data.

[1] The authors are indebted to Dr. T. G. Call, 115 W. Rancho Rd., Corona, Calif., for collection and identification of the plant materials, and to Dr. G. B. Ownbey, Botany Department, University of Minnesota, Minn., for confirmation of the identities. Suitable specimens have been placed in the herbarium of the Botany Department, University of Minnesota.

from the oil deposit which again separated. After this process had been repeated several times, large tetragonal crystals formed on the bottom of the beaker. The crystals were collected in successive crops to yield 15.3 Gm. of crude pteryxin. The resinous, oily deposits were combined, dissolved in boiling Skellysolve B, and treated in the same manner. An additional 4.7 Gm. was obtained for a total of 20 Gm. (2%) of crude pteryxin. Recrystallization from Skellysolve B gave slightly yellow tetragonal prisms melting at 79–81°. The melting point was not depressed on admixture with a sample of pteryxin previously isolated by Call.

A twice recrystallized sample of pteryxin, dissolved in Skellysolve B, was spotted on several chromatostrips.[2] Each chromatostrip was developed in an ascending manner in a stoppered test tube containing about 2 ml. of solvent. Of the several solvent systems tried, only 10% anhydrous ethanol in Skellysolve B separated the sample into four fluorescent spots.

Twice recrystallized pteryxin (500 mg.) dissolved in anhydrous ethanol (5 ml.) was added to the top of a column of silica gel (150 Gm., Davison, commercial grade) prepared in a slurry with 10% anhydrous ethanol in Skellysolve B. Development with the same solvent separated the material into four zones which were eluted. Evaporation of the first two and last fractions gave only trace residues. The third fraction afforded a colorless resin (420 mg.), which was dissolved in boiling Skellysolve B. The solution was filtered, concentrated to about two-thirds its volume, and allowed to stand in a stoppered flask. After several days small colorless prisms began to form. The stopper was removed and the flask covered with a piece of filter paper. After four days, pteryxin crystallized as large, colorless, tetragonal prisms which were filtered off and dried at room temperature in vacuum for several days, m.p. 81.5–82.5°; $[\alpha]_D^{22}$ +10° (c 0.650, C_2H_5OH); ultraviolet spectrum (Fig. 1): $\lambda_{min.}$ 252 (log ϵ 3.57) and 263 mμ (3.20); $\lambda_{max.}$ 218 (4.31) sh., 244 (3.71) sh., 255 (3.60), 300 (3.92) sh., and 323 mμ (4.11); infrared spectrum (Nujol mull) (Fig. 2): 1757–1745 (α-pyrone and α,β-unsatd. ester C=O), 1637 (α-pyrone ring C=C), 1621, 1502 (aromatic C=C), 1587 (aromatic ring with conjugated C=C), 1321, 1107 (α,β-unsatd. ester C—O), 1122 (Ar—O—CR_3), 908 [$(CH_3)_2C$—O], 846 (1,2,3,4-aromatic substitution) and 839 cm.$^{-1}$ (trisubstituted ethylene C—H); (c 2.0, CH_2Cl_2): 1393 and 1377 cm.$^{-1}$ ([$CH_3)_2C$=]. [3] Development of it on a chromatostrip with 10% anhydrous ethanol in Skellysolve B gave a single homogeneous spot.

Anal.[4]—Calcd. for $C_{21}H_{22}O_7$: C, 65.27; H, 5.74; mol. wt., 386.39. Found: C, 66.18; H, 6.11;[5] mol. wt. (Rast), 339.

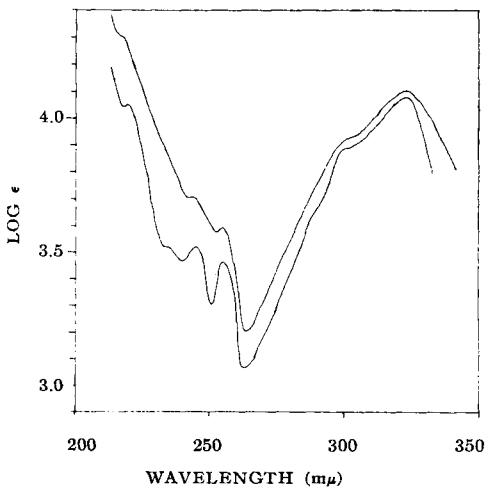


Fig. 1.—Ultraviolet absorption spectra in 95% ethanol. Upper curve, pteryxin; lower curve, suksdorfin.

Isolation of Suksdorfin (II) from *L. Suksdorfii*.—The ground fruit (500 Gm.) was extracted continuously for two days in a Soxhlet apparatus with Skellysolve B (b.p. 65–70°). The colorless crystalline deposit which formed in the concentration flask was filtered off, rinsed with Skellysolve B, and air-dried to give 6 Gm. (1.2%) of suksdorfin, m.p. 130–135°. Five recrystallizations from Skellysolve B afforded fine colorless needles (2 Gm.), m.p. 140–141°. The melting point was not depressed on admixture with a sample of suksdorfin A previously isolated by Call.

A sample of recrystallized suksdorfin was spotted on several chromatostrips which were developed as before, using various solvent systems. Only 30% acetone in Skellysolve B was found to separate a sample into three fluorescent spots.

Recrystallized suksdorfin (0.5 Gm.) was chromatographed on a column of silica gel (150 Gm., Davison, commercial grade) prepared in a slurry with Skellysolve B. Development was conducted with Skellysolve B to which acetone was added gradually (1% increase every 10 ml.) until a concentration of 30% acetone was reached. The major fraction was eluted; the column extruded; and the two remaining zones separated and eluted from the adsorbent with acetone, which was evaporated to give only faint traces. Two recrystallizations of the major fraction from Skellysolve B followed by drying at 100° in vacuum overnight afforded fine colorless needles, m. p. 140.5–141°; $[\alpha]_D^{24}$ + 4° (c 0.500, C_2H_5OH); ultraviolet spectrum (Fig. 1): $\lambda_{min.}$ 240 (log ϵ 3.47), 251 (3.30) and 263 mμ (3.07); $\lambda_{max.}$ 219 (4.05), 234 (3.52) sh., 245 (3.52), 255 (3.46), 300 (3.88) sh., and 323 mμ (4.07); infrared spectrum: (Nujol mull) (Fig. 3): 1754 (ester C=O), 1736 (α-pyrone C=O), 1631 (α-pyrone ring C=C), 1610, 1493, 1445 (aromatic C=C), 1572 (aromatic ring with conjugated C=C), 1250, 1120 (Ar—O—CR_3), 902 [$(CH_3)_2C$—O] and 856 cm.$^{-1}$ (1,2,3,4-aromatic substitution); (c 2.0, CH_2Cl_2): 1395 and 1376 cm.$^{-1}$ [$C(CH_3)_2$]. A sample developed with 30% acetone in Skellysolve B on a chromatostrip showed only one distinct spot. In a volatile acid

[2] The chromatostrips consist of glass strips, 13 × 136 mm., coated on one surface with a thin film of silicic acid containing 5% starch and 0.1% each of zinc silicate and zinc-cadmium sulfide. Their preparation and development procedure is essentially the same as described by Stanley (11).

[3] The assignment of groups responsible for some of the absorption bands in the infrared spectra of the compounds discussed in this paper are in agreement with the findings of Schroeder, *et al.* (4).

[4] Microanalyses were determined by the Microanalytical Laboratory of the School of Chemistry, University of Minnesota, Minneapolis, or by Drs. Weiler and Strauss, 164 Banbury Rd., Oxford, England.

[5] Average percentages. All attempts to prepare a sample which analyzed accurately were unsuccessful.

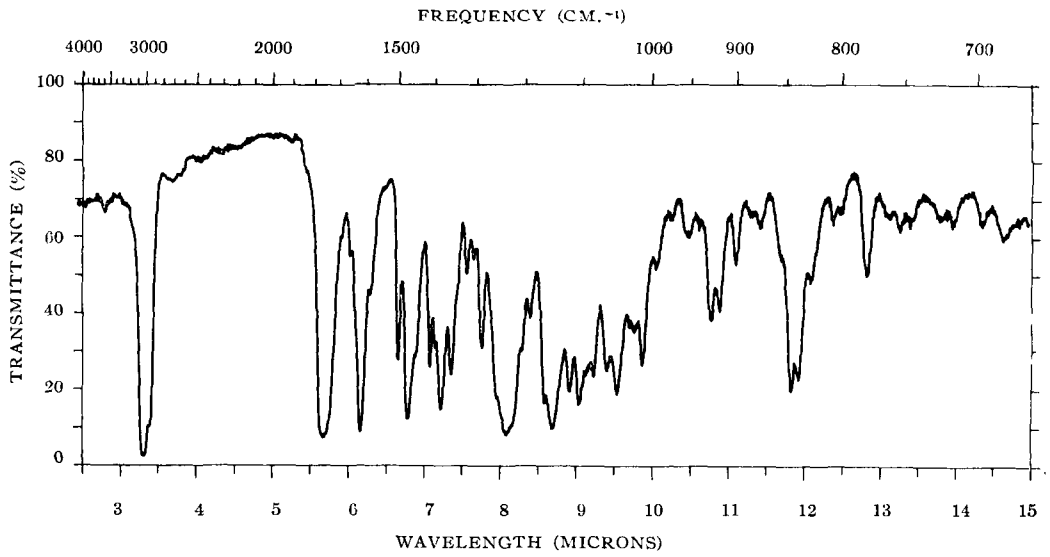


Fig. 2.—Infrared absorption spectrum of pteryxin (Nujol mull).

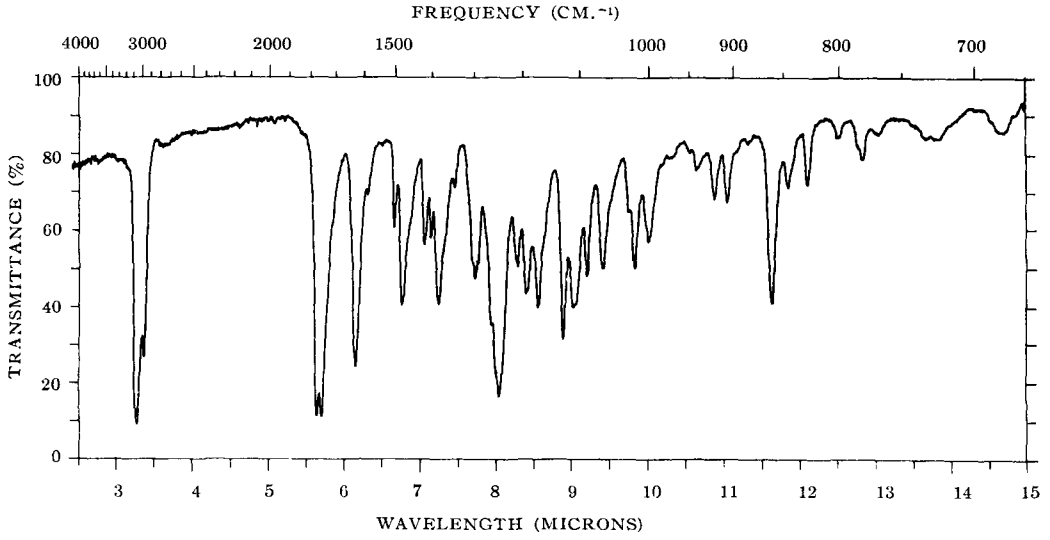


Fig. 3.—Infrared absorption spectrum of suksdorfin (Nujol mull).

determination suksdorfin yielded an amount of acid equivalent to 38.42 ml. of 0.01 *N* sodium hydroxide; calcd. for two acids, 41.89 ml.

Anal.—Calcd. for $C_{21}H_{24}O_7$: C, 64.95; H, 6.23; mol. wt., 388.30. Found: C, 64.88; H, 6.31; mol. wt. (Rast), 357.

Hydrogenation of Pteryxin.—Chromatographically pure pteryxin (77.2 mg., 0.2 mmole) was dissolved in ethanol (10 ml.) and hydrogenated at atmospheric pressure in the presence of platinum oxide (20 mg.). After 20 minutes, 0.17 mmole of hydrogen was consumed, the hydrogenation was interrupted, the catalyst filtered off, and the filtrate evaporated on a steam bath. Two crystallizations of the oily residue from dilute ethanol followed by drying at 78° in vacuum gave dihydropteryxin as fine colorless needles, m.p. 103–104°; ultraviolet spectrum: $\lambda_{min.}$ 262 mμ (log ε 3.20); $\lambda_{max.}$ 218 (4.23) and 322 mμ (4.15); infrared spectrum (Nujol mull): 1172 cm.$^{-1}$ (satd. ester C—O).

Anal.—Calcd. for $C_{21}H_{24}O_7$: C, 64.95; H, 6.23. Found: C, 65.09; H, 6.04.

Chromatographically pure pteryxin (77.2 mg., 0.2 mmole) was treated as above. After 3 hours, 0.36 mmole of hydrogen was consumed, the hydrogenation interrupted, and the product worked up as above. Recrystallization from dilute ethanol and drying for several hours at 100° in vacuum afforded tetrahydropteryxin as fine colorless needles, m.p. 115–115.5°; ultraviolet spectrum: $\lambda_{min.}$ 262 (log ε 2.92) and 301 mμ (3.64); $\lambda_{max.}$ 243 (3.50) sh., 255 (3.22), 284 (3.72) sh., 291 (3.76), and 323 mμ (3.82); infrared spectrum (Nujol mull): 1764 (γ,δ-unsatd. δ-lactone C=O) and 1739 cm.$^{-1}$ (satd. ester C=O).

Anal.—Calcd. for $C_{21}H_{26}O_7$: C, 64.60; H, 6.71. Found: C, 64.21; H, 6.64.

Ethanolic Alkaline Hydrolysis.—Crude pteryxin (15 Gm.), dissolved in 95% ethanol (150 ml.), was mixed with 1 *N* ethanolic sodium hydroxide (150 ml.) and allowed to stand at room temperature for 30 minutes. The mixture was poured into water (2.5 L.), acidified with concentrated sulfuric acid (4 ml.), and extracted with six 500-ml. portions of ether. The combined ether extract was backwashed with three 200-ml. portions of 5% sodium carbonate solution, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue crystallized from dilute ethanol as colorless needles (4.1 Gm.). A sample of it separated into five distinct fluorescent spots on a chromatostrip developed with 50% ethyl acetate in Skellysolve B. Recrystallization from dilute ethanol gave colorless needles (2.5 Gm.) which separated into three spots on a chromatostrip. A portion of these needles (1.0 Gm.) was chromatographed on a column of silica gel (100 Gm., Davison, commercial grade) prepared in a slurry with Skellysolve B. Elution with 20% ethyl acetate in Skellysolve B gave, after recrystallization from dilute ethanol, 0.8 Gm. of colorless needles m.p. 143–144°, which separated into two spots on a chromatostrip. One other zone was eluted in trace amounts.

(+)-*Cis*-**ethylkhellactone (XI).**[6]—The material obtained above (0.8 Gm.) was rechromatographed on a similar column. Development with 10% ethyl acetate in Skellysolve B separated two zones. Elution of the lowest zone with the same solvent gave (+)-*cis*-ethylkhellactone. Repeated crystallizations from dilute ethanol and drying at 78° in vacuum overnight afforded fine colorless needles, m.p. 127–128°; $[\alpha]_D^{24}$ +136° (c 1.00, C_2H_5OH); ultraviolet spectrum: $\lambda_{min.}$ 243 (log ε 3.47), 253 (3.44), and 266 mμ (3.18); $\lambda_{max.}$ 218 (4.12) sh., 247 (3.51), 258 (3.48); 300 (3.93) sh., and 327 mμ (4.19); infrared spectrum (Nujol mull): 3521 (OH), 1716 (α-pyrone C=O), 1626 (α-pyrone ring C=C), 1608, 1490 (aromatic C=C), 1567 (aromatic ring with conjugated C=C), 1342, 1104 (O—H deformation and C—O stretching), 1121 (Ar—O—CR_3), 1057 (ethyl ether), 889 [$(CH_3)_2C$—O] and 838 cm.$^{-1}$ (1,2,3,4-aromatic substitution).

Anal.—Calcd. for $C_{16}H_{18}O_5$: C, 66.19; H, 6.25; mol. wt., 290.30. Found: C, 65.75; H, 6.14; mol. wt. (Rast), 285.

(−)-*Trans*-**ethylkhellactone (IX).**—Further elution of the above column afforded (−)-*trans*-ethylkhellactone. Recrystallization from dilute methanol and drying at 100° in vacuum overnight gave fine colorless needles, m.p. 161–162°; $[\alpha]_D^{23}$ −64° (c 0.5, C_2H_5OH) [lit. m.p. 161–162°, $[\alpha]_D$ −59° (c 0.9, C_2H_5OH) (2)]; ultraviolet spectrum: $\lambda_{min.}$ 266 mμ (log ε 3.18); $\lambda_{max.}$ 327 mμ (4.19); infrared spectrum (Nujol mull) was similar to that of the (+)-*cis*-isomer except for the following major differences: 3636 (OH), 1323 and 1082 cm.$^{-1}$ (OH deformation and C—O stretching.)

The melting point was not depressed on admixture with an authentic sample of (−)-*trans*-ethylkhellactone.[7] Its ultraviolet and infrared spectra were identical to those of the authentic sample. This same compound was obtained by refluxing suksdorfin with 0.5 *N* ethanolic sodium hydroxide for 30 minutes, followed by the same work-up, and purified by repeated crystallizations from dilute methanol.

Anal.—Calcd. for $C_{16}H_{18}O_5$: C, 66.19; H, 6.25; mol. wt., 290.30. Found: C, 66.06; H, 6.40; mol. wt. (Rast), 286.

Isolation and Identification of Acids.—The rapid, horizontal paper chromatographic method of Roberts and Bucek (12) was used to obtain a preliminary characterization of the acids released by hydrolysis. Samples of pteryxin and suksdorfin (25 mg.) were refluxed with 1 *N* sodium hydroxide in 75% ethanol for 30 minutes. The solutions were acidified with concentrated sulfuric acid, adjusted to pH 8 with 33% aqueous ethylamine, and spotted on the paper. After development with a butanol-water solvent system, the acid spots were located by spraying the paper with 0.2% ethanolic chlorophenol red. Pteryxin gave two spots which corresponded quite closely with control samples of acetic and angelic acids, and suksdorfin, with acetic and isovaleric acids.

Pteryxin (1.0 Gm.) was refluxed with 1 *N* ethanolic potassium hydroxide (20 ml.) for 1 hour. The reaction mixture was diluted with water (50 ml.), freed of ethanol under reduced pressure, adjusted to pH 8 with concentrated sulfuric acid, and extracted with ether. The ether extract was evaporated to dryness. The *p*-phenylphenacyl esters were prepared from the residue and chromatographed on a silicic acid-Celite (3:1) column in the manner described by Klohs, *et al.* (13). Elution of the column with 1:1 benzene-petroleum ether (60–110°) yielded in the first fraction unreacted bromide, m.p. 124°. The second fraction afforded colorless crystals which, after recrystallization from ethanol, gave *p*-phenylphenacyl angelate as shiny platelets, m.p. 87–88° [lit. m.p. 90–91° (14)]. The melting point was not depressed on admixture with an authentic sample of *p*-phenylphenacyl angelate.[8]

Anal.—Calcd. for $C_{19}H_{18}O_3$: C, 77.53; H, 6.16. Found: C, 77.54; H, 6.16.

Further elution with 2:1 benzene-petroleum ether (60–110°) yielded colorless needles. Recrystallization from ethanol and drying at 100° in vacuum gave *p*-phenylphenacyl acetate as fine colorless needles, m.p. 109–110° [lit. m.p. 110–111° (13)]. The melting point was not depressed on admixture with the same derivative prepared from acetic acid.

Anal.—Calcd. for $C_{16}H_{14}O_3$: C, 75.58; H, 5.55. Found: C, 75.37; H, 5.73.

The *p*-phenylphenacyl esters of the acids obtained from suksdorfin in the same manner afforded *p*-phenylphenacyl acetate, m.p. 109–110°, no depression on admixture with the authentic sample, and *p*-phenylphenacyl isovalerate as shiny platelets, m.p. 78.5–79° [lit. m.p. 78° (15)]. The melting point was not depressed on admixture with the same derivative prepared from isovaleric acid.

Anal.—Calcd. for $C_{19}H_{20}O_3$: C, 77.01; H, 6.80. Found: C, 76.94; H, 6.94.

(−)-*Cis*-**acetylethylkhellactone (VII).**—A mixture of (+)-*cis*-ethylkhellactone (230 mg.), acetic anhydride (2 ml.), and dry pyridine (2 ml.) was heated on a steam bath for 1 hour and poured into water

[6] The compounds described in this paper have been named according to the scheme followed by Schroeder, *et al.* (4).

[7] Graciously supplied by Dr. Eric Smith, S. B. Penick and Co., New York, N. Y.

[8] Graciously supplied by Dr. S. W. Pelletier, the Rockefeller Institute for Medical Research, New York, N. Y.

(40 ml.). Upon standing overnight fine needles formed and were filtered from the liquor, leaving a clear viscous oil which had separated on the bottom of the beaker. The needles were recrystallized twice from dilute methanol and dried at 78° in vacuum to give (−)-*cis*-acetylethylkhellactone as colorless prisms, m.p. 122–123°; $[\alpha]_D^{25}$ −15° (c 0.527, C_2H_5OH); ultraviolet spectrum: $\lambda_{min.}$ 263 mμ (log ε 3.20); $\lambda_{max.}$ 325 mμ (4.18); infrared spectrum (Nujol mull): 1247 and 1043 cm.$^{-1}$ (acetate C—O).

Anal.—Calcd. for $C_{18}H_{20}O_6$: C, 65.05; H, 6.07. Found: C, 64.67; H, 6.26.

(−)-*Trans*-**acetylethylkhellactone (V).**—The residual oil obtained above crystallized upon rubbing with methanol. Three recrystallizations from dilute methanol followed by drying at 78° in vacuum gave (−)-*trans*-acetylethylkhellactone as colorless needles, m.p. 168–168.5°; $[\alpha]_D^{24}$ −39° (c 1.77, C_2H_5OH) [lit. m.p. 170–172°, $[\alpha]_D$ −40.6° (c 0.6, C_2H_5OH) (2)]; ultraviolet spectrum: $\lambda_{min.}$ 264 mμ (log ε 3.26); $\lambda_{max.}$ 325 mμ (4.20); infrared spectrum (Nujol mull): 1245 and 1042 cm.$^{-1}$ (acetate C—O).

Anal.—Calcd. for $C_{18}H_{20}O_6$: C, 65.05; H, 6.07. Found: C, 64.90; H, 6.14.

This acetate was obtained from (−)-*trans*-ethylkhellactone in the same manner.

Methanolic Alkaline Hydrolysis.—Crude pteryxin (15 Gm.) dissolved in methanol (60 ml.) was mixed with 1 *N* methanolic sodium hydroxide (60 ml.) and refluxed 90 minutes. The brown mixture was diluted with water (300 ml.) and the methanol removed under reduced pressure. The aqueous solution was extracted with three 100-ml. portions of methylene chloride, which were combined and evaporated to dryness. The light brown residue (4.0 Gm.) was recrystallized from methanol, then ether-Skellysolve B as colorless needles (2.5 Gm.), which separated into two spots on a chromatostrip developed with 50% ethyl acetate in Skellysolve B; m.p. 159–160°; $[\alpha]_D^{24}$ +37° (c 1.00, CH_3OH), corresponding to a mixture of 40% (+)-*cis*- and 60% (−)-*trans*-methylkhellactone.[9]

(+)-*Cis*-**methylkhellactone (XII).**—The above mixture (731 mg.) was chromatographed on neutral alumina (25 Gm., Woelm, activity III, prepared according to Brockmann). Elution with benzene gave (+)-*cis*-methylkhellactone (295 mg.), m.p. 126–127°; $[\alpha]_D^{23}$ +88° (c 0.752, C_2H_5OH) [lit. m.p. 127°, $[\alpha]_D^{22}$ +97° (c 0.628, CH_3OH) (4)]; ultraviolet spectrum: $\lambda_{min.}$ 266 mμ (log ε 3.18); $\lambda_{max.}$ 329 mμ (4.21); infrared spectrum (Nujol mull): 3521 (OH), 1351 and 1111 cm.$^{-1}$ (OH deformation and C—O stretching).

Anal.—Calcd. for $C_{15}H_{16}O_5$: C, 65.19; H, 5.84. Found: C, 65.20; H, 6.01.

(−)-*Trans*-**methylkhellactone (X).**—Further elution of the above column with 1:4 ether-benzene gave (−)-*trans*-methylkhellactone (313 mg.) which was recrystallized from ether-petroleum ether (30–60°) as colorless needles, m.p. 160–161°; $[\alpha]_D^{25}$ − 8° (c 0.793, C_2H_5OH) [lit. m.p. 161.5–162.5°, $[\alpha]_D^{24}$ −3° (c 1.374, CH_3OH) (4)]; ultraviolet spectrum: $\lambda_{min.}$ 265 mμ (log ε 3.32); $\lambda_{max.}$ 328 mμ (4.21); infrared spectrum (Nujol mull): 3571 (OH), 1328 and 1101 cm.$^{-1}$ (OH deformation and C—O stretching).

Anal.—Calcd. for $C_{15}H_{16}O_5$: C, 65.19; H, 5.84. Found: C, 65.44; H, 6.03.

On acetylation, the methyl ether mixture (300 mg.) gave, after recrystallization from dilute methanol and drying at 100° in vacuum, (+)-*trans*-acetylmethylkhellactone (VI) as colorless needles (200 mg.), m.p. 148–149° [lit. m.p. 152.5° (4)]; ultraviolet spectrum: $\lambda_{min.}$ 263 mμ (log ε 3.14); $\lambda_{max.}$ 325 mμ (4.18).

Anal.—Calcd. for $C_{17}H_{18}O_6$: C, 64.14; H, 5.70. Found: C, 64.14; H, 5.68.

Quantitative Hydrolytic Experiments.—Samples of recrystallized pteryxin (61.8 mg.), suksdorfin (61.4 mg.), visnadin[10] (51.5 mg.), and (+)-*trans*-acetylmethylkhellactone were each dissolved in 0.25 *N* ethanolic sodium hydroxide (10.0 ml.) at room temperature. Aliquots (1.0 ml.) taken at various intervals were diluted with carbon dioxide-free water (15 ml.) and titrated with 0.0243 *N* sulfuric acid to pH 7 on a Beckman Zeromatic pH meter. A blank determination of the alkali solution was made at the same time. The results expressed as mole equivalents plotted against time in minutes are shown in Fig. 4.

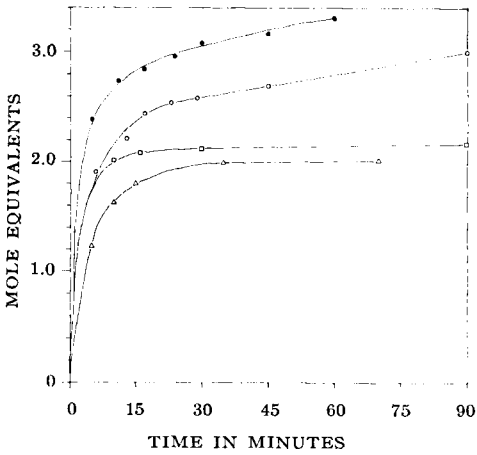


Fig. 4.—Hydrolysis rates in 0.25 *N* ethanolic sodium hydroxide at room temperature. ●, suksdorfin; ○, pteryxin; □, visnadin; △, (+)-*trans*-acetylmethylkhellactone.

Controlled Hydrolysis of Pteryxin.—Recrystallized pteryxin (1.0 Gm., 2.6 mmole) was dissolved in 0.25 *N* ethanolic sodium hydroxide (25.0 ml.) at room temperature and the rotation taken every 30 seconds. The rotation decreased rapidly for 8 minutes, remained constant for the next 4 minutes, and then started to decrease. An aliquot (1.0 ml.) was taken quickly and added to water (15 ml.) as the remaining solution was simultaneously added to an equivalent amount of sulfuric acid (6 meq.) in water (250 ml.). The reaction period was 13 minutes. The diluted aliquot was titrated im-

[9] It should be pointed out that Smith, *et al.* (2), obtained from samidin a methyl ether which melted at 158–159° and gave a rotation of $[\alpha]_D$ +14°, which is a mixture of approximately 83% *trans*- and 17% *cis*-isomers. Schroeder, *et al.* (4), have drawn attention to the fact that in no case did these workers report isomeric pairs, even though the isomers had been previously characterized in a preliminary report by the Swiss workers (16).

[10] Graciously supplied by Dr. Hamed Abu-Shady, Pharm. Chem. Department, Cairo University, Cairo, Egypt.

mediately with 0.0243 *N* sulfuric acid; a blank of the alkali solution was titrated at the same time. The alkali consumed was 1.75 mole equivalents. The aqueous acid solution of the reaction mixture was extracted with five 25-ml. portions of 3:1 ether-methylene chloride. The combined ethereal extract was washed with 10% sodium bicarbonate solution followed by saturated sodium chloride solution. The washed extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dried to constant weight in vacuum (680 mg.) and chromatographed on a column of neutral alumina (15 Gm., Woelm, Brockmann activity III). Elution with benzene yielded a crystalline residue from the first fraction (20 ml.). Recrystallization from methanol and drying at 78° in vacuum overnight afforded (−)-*trans*-acetylethylkhellactone (V), m.p. 167–168°. The melting point was not depressed on admixture with the acetate prepared from (−)-*trans*-ethylkhellactone. Their infrared spectra were superimposable.

Anal.—Calcd. for $C_{18}H_{20}O_6$: C, 65.05; H, 6.07. Found: C, 64.90; H, 6.15.

Continued elution with benzene, after removal of a dark zone, yielded a second crystalline fraction which was recrystallized from dilute methanol and dried at 78° in vacuum to give (+)-*cis*-ethylkhellactone (XI) as very fine colorless needles, m.p. 126–127°. The melting point was not depressed on admixture with a sample of (+)-*cis*-ethylkhellactone obtained previously from pteryxin. Their infrared spectra were identical.

Anal.—Calcd. for $C_{16}H_{18}O_5$: C, 66.19; H, 6.25. Found: C, 66.17; H, 6.36.

Further elution afforded only trace amounts of material.

Controlled Hydrolysis of Suksdorfin.—Suksdorfin (1.00 Gm., 2.58 mmole) was hydrolyzed in the same manner as above. The reaction period was 5 minutes with 1.65 mole equivalents of alkali being consumed. The reaction mixture was worked-up and the residue chromatographed as above. (−)-*Trans*-acetylethylkhellactone, m.p. 169–170°, and (+)-*cis*-ethylkhellactone, m.p. 127–128°, were obtained. Mixed melting points with samples obtained previously showed no depression and their infrared spectra were identical. Further elution of the column afforded only trace amounts of material.

3′-Keto-3′,4′-dihydroseselin (XIII).—Ethylkhellactone (2.18 Gm.) was treated with *p*-toluenesulfonic acid in boiling benzene in the same manner described by Schroeder, *et al.* (4). Recrystallization from 1:1 acetone-Skellysolve B gave 1.08 Gm. (59%) of light yellow prisms, m.p. 157–157.5° [lit. m.p. 156.5–157.5° (4)]; ultraviolet spectrum: $\lambda_{min.}$ 263 mμ (log ε 3.40); $\lambda_{max.}$ 321 mμ (4.10); infrared spectrum (Nujol mull): 1757 cm.$^{-1}$ (α- and β-pyrone C=O).

Anal.—Calcd. for $C_{14}H_{12}O_4$: C, 68.86; H, 4.95. Found: C, 68.65; H, 4.99.

(±)-3′-Hydroxy-3′,4′-dihydroseselin (XIV).—To a stirred solution of 3′-keto-3′,4′-dihydroseselin (488 mg.) in methanol (40 ml.), cooled to −10° in an ice-salt mixture, was added dropwise an ice-cold solution of sodium borohydride (530 mg.) in methanol (10 ml.) over a 15-minute period. The mixture was stirred an additional 45 minutes at room temperature. The yellow solution was made slightly acidic with 1 *N* hydrochloric acid and concentrated to 10 ml. Upon standing overnight fine red needles formed which were filtered off and air-dried to yield 422 mg. (86%) of product. Repeated crystallizations from dilute ethanol gave pale pink needles, m.p. 165–166°; infrared spectrum (Nujol mull): 3636 (free OH), 1720 (C=O), 1295 and 1090 cm.$^{-1}$ (O—H deformation and C—O stretching).

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.00; H, 5.60.

Acetylation of 200 mg. of the alcohol XIV with acetic anhydride and pyridine gave 220 mg. (94%) of acetate. Recrystallization from dilute ethanol gave fine colorless needles, m.p. 172–173°; infrared spectrum (Nujol mull): 1752–1745 (α-pyrone and acetate C=O) and 1242 cm.$^{-1}$ (acetate C—O).

Anal.—Calcd. for $C_{16}H_{16}O_5$: C, 66.65; H, 5.59. Found: C, 66.50; H, 5.70.

The nuclear magnetic resonance (n.m.r.) spectra of the alcohol XIV and its acetate is in complete agreement with the assigned structures.[11]

DISCUSSION

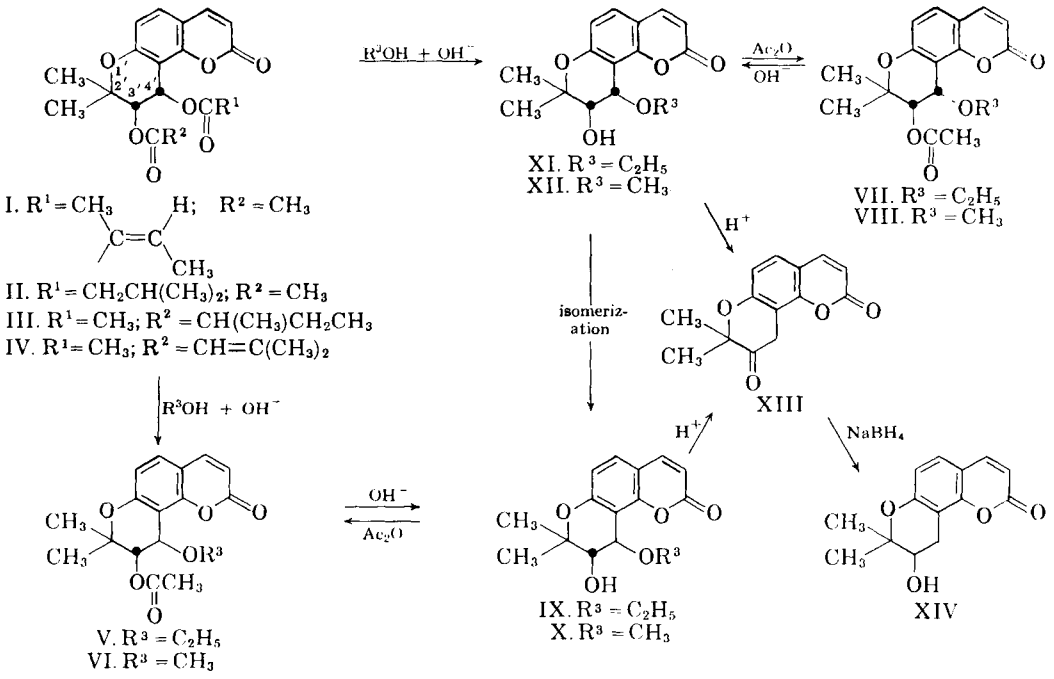
Pteryxin (I) was isolated in 2% yield from dried roots of *P. terebinthina* and suksdorfin (II) in 1.2% yield from dried fruits of *L. Suksdorfii*. Chromatostrips were utilized in finding a satisfactory solvent system to purify these substances. Results obtained on the chromatostrips were readily adaptable to column chromatography and were a definite labor-saving device.

Ultraviolet spectra of these substances and their derivatives had absorption maxima at 322–329 mμ and minima at 263–266 mμ, which are in agreement with their assigned coumarin structures (2).

Upon hydrolysis with ethanolic alkali, pteryxin (I) and suksdorfin (II) both yielded the same isomeric alcohols, (−)-*trans*-ethylkhellactone (IX), which was shown to be identical to the hydrolysate obtained from samidin by Smith, *et al.* (2), and (+)-*cis*-ethylkhellactone (XI). In methanol, the corresponding methyl ethers (X and XII) were obtained. Absolute configurational formulas for the methylkhellactones were established by Schroeder, *et al.* (4). These ethers arise by means of a solvolysis of the 4′-position in the parent compounds, a finding substantiated by both Smith (2) and Schroeder (4). The 4′-position constitutes a benzyl ester, certain ones of which are known to undergo solvolysis with O-alkyl cleavage under acidic or basic conditions (17).

(+)-*Cis*-ethylkhellactone (XI) and its acetate (VII) have not been reported previously. We assigned absolute configurational structures to them on the basis of similarities to the established methyl ethers. The *cis* relationship of the adjacent hydroxyl and ethoxyl groups is indicated by the hydrogen bonding of the hydroxyl group evidenced in its infrared spectrum (3521 cm.$^{-1}$) as compared to the free hydroxyl shown by the *trans*-isomer (3636 cm.$^{-1}$). Additional evidence for hydrogen bonding in the *cis*-isomer is the location of its secondary hydroxyl OH deformation and C—O stretching absorption at 1342 and 1104 cm.$^{-1}$, whereas these peaks are at 1323 and 1082 cm.$^{-1}$ for the *trans*-

[11] The authors thank Mr. George Slomp, The Upjohn Co. Kalamazoo, Mich., for the determination and interpretation of the n.m.r. spectra.



isomer. Bonded hydroxyls are known to absorb at lower wavelengths (18). The same is true of the methyl ethers.

Ethylkhellactone (IX + XI) was dehydrated to form the ketone XIII, which was reduced with sodium borohydride to the alcohol XIV. The structures for alcohol XIV and its acetate were confirmed by nuclear magnetic resonance spectra.

Hydrolysis of pteryxin yielded angelic and acetic acids, and suksdorfin yielded isovaleric and acetic acids. These were characterized by paper chromatography and through their *p*-phenylphenacyl ester derivatives.

In a titration experiment measuring the rate of alkali consumption of visnadin (III) and samidin (IV) during room temperature hydrolysis in ethanolic alkali, Smith, *et al.* (2), reported these coumarins to consume only approximately two equivalents of alkali after 90 minutes. The third equivalent was consumed after refluxing. Also, they were able to isolate a room temperature hydrolysate which contained a five carbon acid moiety at the 3′-position. In a similar experiment, pteryxin (I) consumed all three equivalents at room temperature in 90 minutes, and suksdorfin (II) in 30 minutes.

Esters of α,β-unsaturated acids and higher saturated fatty acids hydrolyze in acid or base at a significantly slower rate than acetates (19). Clearly, the α,β-unsaturated angeloyl group in pteryxin and the isovaleryl group in suksdorfin are not on the 3′-oxygen, as is the case in visnadin and samidin, but are on the 4′-oxygen, where the solvolysis rate is not affected by the nature of the acid substituent. It follows therefore, that the acetoxy group is at the 3′-position and, inasmuch as it offers less resistance to hydrolysis, is cleaved at room temperature in a relatively short period of time.

If the assignment of the acetoxy group to the 3′-position is correct, then the acetate of the intermediate ether (V or VII) should hydrolyze at a similar rate. Such was the case. Hydrolysis of (+)-*trans*-acetylmethylkhellactone (VI) was complete in 35 minutes.

By quenching the hydrolysis of the parent coumarins after 5 to 13 minutes, a mixture of (+)-*trans*-ethylkhellactone acetate (V) and (+)-*cis*-ethylkhellactone (XI) was obtained. Isolation of the intermediate acetate (V), which was identical to that prepared from the alcohol, conclusively proves pteryxin and suksdorfin to have structures I and II, respectively.

Migration of the acyl or acyloxy group from the 4′-position, to the 3′-position, which were arbitrarily designated as *trans*, was discussed by Smith, *et al.* (2), and the possibility eliminated. Establishment of their *cis* configuration makes a migration even more remote.

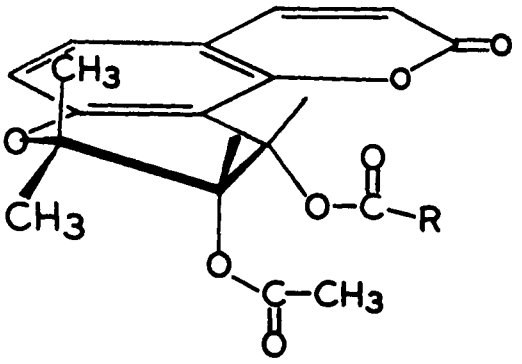
The hydrolytic studies indicated that the 3′-acetyl group was being hydrolyzed concomitantly, although at a slower rate, with the loss of the angeloyloxy group. As indicated above, the partial hydrolysis product was the *trans*-acetate (V) and the initial completely hydrolyzed one the *cis*-alcohol XI. If the reaction was allowed to proceed for an additional 15 minutes, the major product was the *trans*-alcohol IX, which arises by hydrolysis of the acetate V and by isomerization of the less stable *cis*-alcohol XI.

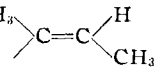
The hydrolytic results described above may be rationalized on the basis that the system is undergoing two different competitive processes, the one being kinetically controlled and relatively fast and the other being a slower equilibrium process. The former leads to the less stable *cis*-isomer and the latter predominantly to the more stable *trans*-isomer. It may be considered that the process, as postulated by Smith, *et al.* (2), involves first a fast S_N1 type expulsion of the 4′-acyloxy group initiated by the neighboring phenolic group. This leads to an enone intermediate which stabilizes the resulting carbonium ion. A study of structural models of the species involved leads us to suggest that the results of the

hydrolytic studies may be explained plausibly if one considers that the 3′-acetoxy group is undergoing continuous hydrolysis from the moment it is subjected to alkaline conditions. Thus, at all times, we can postulate two species of carbonium ion present, one with an acetoxy group and one with only a hydroxyl group at the 3′-carbon atom. The arrangement of groups on this carbon atom offers the strong possibility of a neighboring group type participation between the quasi-axially oriented acetoxy carbonyl and the carbonium ion center. Stabilization of the acetoxy group in this manner would reasonably promote ethoxide ion addition on the less hindered *trans*-side and lead to the *trans*-acetate V. On the other hand, the alcoholic species would no longer have this stabilizing influence and, therefore, both *cis*- and *trans*-addition can be envisioned. In this respect, the *cis*-addition, favored by a serious axial methyl (at the 2′-position) interference with *trans*-addition, is suggested as being quite rapid kinetically and would account for the isolation of the *cis*-alcohol XI when the reaction is quenched before equilibrium has been achieved. As the process proceeds, however, equilibration finally results in a preponderance of the *trans*-alcohol IX over the *cis*-alcohol, the former being obtained by hydrolysis of V as well as by isomerization of the less stable XI under alkaline conditions.

Pteryxin (I) and suksdorfin (II) have the same configuration as visnadin (III) and samidin (IV) at the 3′-position, as evidenced by the formation of the same hydrolytic products. The absolute configurational structures for the latter pair of compounds were established by Schroeder, *et al.* (4), on the basis of their (+)-rotation and the similarities of their infrared spectra in the 9 and 10 μ region to synthetic (±)-*cis*-diacetylkhellactone. Also, these infrared spectra differed significantly from those of synthetic (±)-*trans*-samidin and (±)-*trans*-diacetylkhellactone. Since pteryxin (I) and suksdorfin (II) possess these features too, they have the same configuration as visnadin (III) and samidin (IV) at the 4′-position, and are, therefore, established as (+)-*cis*-pteryxin and (+)-*cis*-suksdorfin.

We propose the following absolute conformational structures for (+)-*cis*-pteryxin (XV) and (+)-*cis*-suksdorfin (XVI) with the 3′-acetoxy and 4′-acyloxy groups in axial and quasi-equatorial positions, respectively. The arrangement of these two groups in molecular models is distorted from the normal axial and equatorial positions that would be encountered if the ring system were a cyclohexane type rather than the fused dihydro-pyrano system that actually exists. This, no doubt, is due to the different bond lengths and angles of the ether oxygen.



XV: R = 

XVI: R = $CH_2CH(CH_3)_2$

The mechanisms involved in the hydrolysis of these coumarin esters and additional stereochemical proofs are presently under further investigation.

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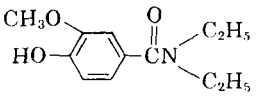
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Central Stimulatory Activity of Vanillic Diethylamide

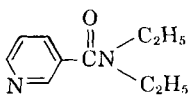
By JOSEPH P. BUCKLEY, MARIO D. G. ACETO, and WILLIAM J. KINNARD

Vanillic diethylamide produced central nervous system effects indicative of medullary stimulation when injected into frogs and rats. Vanillic diethylamide administered intravenously to anesthetized dogs and cats produced a marked increase in both the rate and depth of respiration, which was usually accompanied by the return of the corneal reflex and a mild, transient hypotensive effect. The transient hypotensive effect could be eliminated by administering the compound via a slow infusion at rates ranging between 1 and 8 mg./Kg./min. The compound produced identical responses prior to and after bilateral carotid sinus area denervation, and it appears that the compound acts mainly on the medullary centers and that the carotid sinus-body complex is not essential for the stimulatory activity. A 10 mg./Kg. quantity of vanillic diethylamide administered into the arterial inflow to the recipient's head in 2 dog cross-circulation experiments awakened the recipient animal and produced a transient pressor effect in the recipient.

IT HAS BEEN known for many years that the analeptic activity of the alkylated acid amides was due to the alkylation of the nitrogen of the acid amide group (1). The clinical success with nikethamide, an active member of the alkylated acid amide group, prompted further synthesis of related compounds. Vanillic diethylamide, 3-methoxy-4-oxybenzoic acid-diethylamide (see Fig. 1), was prepared and tested on experimental animals in an attempt to produce a more potent compound (2, 3). The object of the present study was to investigate certain central stimulatory actions of the compound and to study its possible sites of action.



Vanillic Diethylamide



Nikethamide

Fig. 1.—Chemical structure of vanillic diethylamide and nikethamide.

Received April 28, 1961, from the Department of Pharmacology, University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.

Accepted for publication June 22, 1961.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

Supported in part by a research grant from U. S. Vitamin & Pharmaceutical Corp., New York, N. Y.

EXPERIMENTAL PROCEDURES

Preliminary CNS Screening in Frogs.—Doses of vanillic diethylamide, ranging between 10 and 50 mg./Kg., were administered into the ventral lymph sac of 12 frogs. General activity was noted and if hyperreflexia or convulsions occurred, either decerebration or decerebration and demedullation was performed.

Preliminary CNS Screening in Rats.—An aqueous solution of vanillic diethylamide was administered intraperitoneally to 16 rats in doses ranging from 25 to 75 mg./Kg., and the gross behavior of the animals recorded.

Effect on Anesthetized Dogs and Cats.—Mongrel dogs were anesthetized with an intravenous dose of 35 mg./Kg. of sodium pentobarbital. Cats were anesthetized by an intraperitoneal injection of the same dose of sodium pentobarbital. Blood pressure was recorded from a carotid or femoral artery via a Statham transducer, and respiration was recorded from a thermocouple wire in the tracheal cannula. The neural plexi surrounding both carotid sinuses and carotid bodies were stripped in three experiments. The elimination of the pressor response to bilateral carotid occlusion was utilized as one of the criteria for complete denervation of the carotid sinus area. Physiological responses were recorded on a Grass polygraph. Fresh aqueous solutions of vanillic diethylamide were administered intravenously in doses ranging from 2.5 to 25 mg./Kg.

Effect on Dogs and Cats under Severe Barbiturate Depression.—Dogs and cats were anesthetized and prepared for the recording of blood pressure and respiration as previously noted. The animals were further depressed by intravenous doses of sodium pentobarbital. At a point of severe respiratory depression, vanillic diethylamide was administered intravenously in an aqueous solution.

Effect of Vanillic Diethylamide Infusion on Depressed Cats.—Cats were anesthetized by an intraperitoneal injection of 35 mg./Kg. of sodium pentobarbital. Physiological recordings were made as previously noted. Vanillic diethylamide was infused intravenously into the depressed animal utilizing a microdrip apparatus.[1] Bilateral carotid

[1] Venopak microdrip, Abbott Laboratories.

sinus-body denervation was produced in three preparations.

Central Effects of Vanillic Diethylamide.—The central effects of vanillic diethylamide were investigated in two dog cross-circulation preparations. Mongrel dogs were anesthetized by an intravenous injection of pentobarbital sodium, 35 mg./Kg., and the vascularly isolated, neurally intact recipient head preparation was obtained utilizing the procedure described by Buckley, *et al.* (4). Donor and recipient femoral blood pressures were obtained, and electroencephalographic tracings were obtained from frontal electrodes in the recipient. Vanillic diethylamide, 10 mg./Kg., was administered in aqueous solution via the carotid artery to the recipient's head.

RESULTS

Preliminary CNS Screening in Frogs.—Doses of 10 and 25 mg./Kg. of vanillic diethylamide produced hyperreflexia which disappeared upon decerebration. Injection of 50 mg./Kg. produced clonic convulsions which were abolished by demedullation.

Preliminary CNS Screening in Rats.—The effects of vanillic diethylamide, in doses ranging between 25 and 75 mg./Kg., i.p., on the activity of albino Wistar rats are summarized in Table I and indicate that the compound is a potent stimulant of the central nervous system. The type of convulsions produced by the larger doses suggests the medulla as the possible site of stimulation.

TABLE I.—EFFECTS OF VANILLIC DIETHYLAMIDE ON ALBINO RATS

No. of Animals	Dose, i.p., mg./Kg.	Results
3	25	No effect
9	50	2 showed slight stimulation, 4 showed extreme excitement short of convulsions, 3 showed clonic convulsions and death within 5 minutes
4	75	3 showed mixed convulsions and death within 5 minutes, 1 showed slight stimulation

Effect on Anesthetized Dogs and Cats.—The intravenous administration of vanillic diethylamide, in the dose range of 10 to 25 mg./Kg., to dogs and cats produced general signs of central nervous system stimulation (See Table II). The common picture of initial apnea lasting 60 seconds or less followed by an increased rate and/or depth of respiration was observed in most of the experiments. The initial hypotensive effect followed by the return to the predrug level or slightly above that level was also seen in the majority of the experiments. Doses of 20 and 25 mg./Kg., i.v., into cats produced signs of arousal. The intravenous injection of vanillic diethylamide to three dogs having bilateral denervation of the carotid sinus-body complex produced the same physiological responses that were seen in animals having intact sinus areas. The administration of 10 mg./Kg. of vanillic diethylamide to the first dog produced a transient fall in blood pressure from 245/135 mm. Hg to 180/80 mm. Hg with a return to control levels within 2 minutes. The respiration was increased, following a transient apnea, to 17 per minute from an initial rate of 13 per minute. The blood pressure in the second dog showed a transient fall from 250/128 mm. Hg to 150/80 mm. Hg and a recovery to 195/120 mm. Hg within 80 seconds following the intravenous injection of 15 mg./Kg. of vanillic diethylamide. The respiration, following a short period of apnea, increased to 18 per minute from the control value of 4 per minute; the respiratory rate then slowed gradually to 7 per minute. The third dog showed an increase in respiratory rate to 63 per minute from the control level of 47 per minute following 10 mg./Kg. of the drug, i.v. The blood pressure followed the same pattern as was seen in the previous dogs.

Effect on Dogs and Cats under Severe Barbiturate Depression.—Vanillic diethylamide, in doses from 5 to 25 mg./Kg., produced in all experiments but one an increase in the rate of respiration (See Table III). Doses of 25 mg./Kg., i.v., in the cat generally produced signs of arousal from the anesthesia. Many of the cardiovascular responses were that of initial transient hypotension with a recovery to near normal levels of blood pressure within a few minutes.

Effects of Vanillic Diethylamide Infusion on Depressed Cats.—The administration of vanillic

TABLE II.—EFFECT OF VANILLIC DIETHYLAMIDE ON ANESTHETIZED[a] DOGS AND CATS

Animal	Initial Blood Pressure, mm. Hg	Initial Respiration, rate/min.	Dose, i.v., mg./Kg.	Effects	
				Respiration	Blood Pressure,[b] mm. Hg
Dog	190/135	18	2.5	17/min. with increased depth	205/145
Dog	250/140	72	10.0	Rate drop to 66/min., depth increased by 50%	Transient fall, then maintained at 260/150
Dog	130/75	31	10.0	Short period of apnea followed by increased rate and depth	Transient fall and then recovery
Dog	175/115	28	10.0	Initial apnea, then increased to 36/min.	Transient fall, followed by a slight hypertensive effect
Dog	200/120	5	15.0	Apnea for 60 sec., rate increased to 12/min., then back to normal in 4 min.	Fall to 104/42, then rise to 175/100 in 80 sec.
Cat	175/130	24	5.0	No change	No change
Cat	75/55	12	10.0	Transient increase	No change
Cat	193/140	19	20.0	Increased to 40/min.	Increased 25%, animal awoke
Cat	145/100	26	25.0	Increased to 50/min.	No change, motor activity of limbs

[a] Anesthetic—pentobarbital sodium, 35 mg./Kg., i.v., for dogs, i.p., for cats. [b] Motor activity also noted.

TABLE III.—EFFECT OF VANILLIC DIETHYLAMIDE ON DOGS AND CATS UNDER DEEP PENTOBARBITAL DEPRESSION

Animal	Additional Doses of Sodium Pentobarbital,[a] mg./Kg.	Blood Pressure, mm. Hg	Respiration, rate/min.	Dose, i.v., mg./Kg.	Effects	
					Blood Pressure, mm. Hg	Respiration,[b] rate/min.
Dog	22.5	155/105	2	10.0	Transient fall then rise to 175/120	Increased to 33
Cat	15.0	...	2–3	5.0	Transient fall then increased to 180/125	Increased to 11 with increase in depth
Cat	7.5	100/75	Stopped	5.0	Increased to 175/125	Increased to 10
Cat	11.0	128/80	20	6.0	Fall to 100/68	Transient apnea, then 15
Cat	9.0	147/108	15	10.0	Transient fall to 115/53 then returned to 151/113 in 3 min.	Increased to 21 for 1 min., then returned to 15
Cat	6.5	...	20	25.0	.....	Extremely rapid with convulsions
Cat	15.0	75/60	Stopped	25.0	160/100	Increased to 15 for 5 min., then stabilized at 8, then corneal reflex positive
Cat	18.0	...	2–3	25.0	Increased	Increased to 15

[a] Dogs anesthetized with pentobarbital sodium, 35 mg./Kg., i.v.; cats anesthetized with pentobarbital sodium, 35 mg./Kg. i.p. [b] Motor activity also noted.

TABLE IV.—EFFECT OF THE INTRAVENOUS INFUSION OF VANILLIC DIETHYLAMIDE ON ANESTHETIZED CATS[a]

Animal	Infusion Rate, mg./Kg./min.	Total Dose, mg./Kg.	Time, min.	Blood Pressure, mm. Hg	Respiration, rate/min.	Motor Activity
A	1.0	89.0	0	200/135	8	.....
			43[b]	240/165	12	.....
			60	...	..	Head movements
			89[c]	225/150	15	Corneal reflex
			99	...	14	.....
			109	...	..	Corneal reflex negative
			129	190/130	13	.....
B	7.8	80.8	0	138/100	17	.....
			6	Irregular	20	Ear twitch
			8.6	Irregular	7.8	Wink reflex
			10.2[c]	Irregular	..	Corneal reflex positive
			12.0	200/120	..	Convulsion
			17.0	...	..	Corneal reflex negative
			30.0	175/125	9	.....
C	3.5	36.2	0	104/68	8	.....
			5[b]			
			10.3[c]	140/83	Slowed and irregular	Ear twitch
			17	175/100	10	.....
D[d]	1.8	56.9	0	172/125	15	.....
			10[b]	194/125	17	Ear twitch
			25	...	12	Corneal reflex positive
			31.6[c]	160/195	9	.....
			35	...	..	Corneal reflex negative
			50	184/121	15	.....
E[d]	1.8	61.7	0	130/90	Slowed and irregular	Wink reflex positive, corneal reflex negative
			8	140/95	8	Corneal reflex positive
			14[b]	160/100	..	Ear twitch
			34.3[c]	123/75	25 shallow	.....
			42	160/92	Slowed with greater depth	.....
F[d]	1.0	30.0	0[b]	205/145	7	.....
			7	Falling	8	Wink reflex positive
			16	Falling	9	Ear twitch
			18	Falling	9	Corneal reflex positive
			30[c]	175/125	7–8	.....
			35	175/125	15	Ear twitch stopped

[a] Anesthetic, pentobarbital sodium, 35 mg./Kg., i.p. [b] Peak hypertensive effect. [c] Cessation of infusion. [d] Bilateral carotid sinus-body denervation.

diethylamide by slow infusion produced pronounced CNS stimulatory effects in anesthetized cats, even when the carotid sinus-body complexes were denervated (See Table IV). In most cases, the blood pressure was elevated during initial stages of the infusion and then gradually fell after a period of time. When the infusion was stopped, arterial blood pressure usually increased or remained stable. The

peak hypertensive response usually occurred very close to the onset of visible motor movements. Since the arterial pressure began to drop shortly after motor activity was observed, it is entirely possible that pain stimuli might be responsible for this secondary depressor response. The respiratory rate generally increased during the initial phase of the infusion and then gradually decreased. The rate of respiration immediately increased when the infusion was stopped. The infusion of vanillic diethylamide into denervated cats produced the same effects as those seen during the infusion of the drug into cats having intact sinus areas (See Table IV).

Central Effects of Vanillic Diethylamide.—The administration of 10 mg./Kg. of vanillic diethylamide into the arterial inflow to the recipient's head in two cross-circulation experiments produced nearly complete arousal of the animals. The recipient animals blinked their eyes, wagged their tails, and attempted to move their heads. An initial apnea occurred in the recipient dogs which was followed by a resumption of respiration that was thoracic in nature as compared to the abdominal breathing observed prior to drug administration. The compound produced an initial hypertensive effect in the recipient dogs. There was little change in the blood pressure and respiration of the donor dogs although there was a slight transient hypotensive response in one dog. An electroencephalogram, obtained from frontal electrodes in the recipient dog, illustrated an increased activity immediately after the administration of vanillic diethylamide. Major analeptic effects were not seen in the donor dog.

DISCUSSION

The parenteral administration of vanillic diethylamide into frogs, rats, cats, and dogs produced definite signs of central nervous system stimulation. Injection of 50 mg./Kg. of the drug into the ventral lymph sac of frogs produced clonic convulsions which were abolished by demedullation, while 50 and 75 mg./Kg.-doses injected in rats produced tonic-clonic convulsions. Single doses of 5 to 25 mg./Kg. administered intravenously into anesthetized and deeply anesthetized dogs and cats generally produced a decrease in the depth of anesthesia. Following the injection of the drug, an initial phase of apnea and hypotension usually occurred; the respiration then increased in rate and/or depth while the blood pressure returned to normal or slightly hypertensive levels. Nikethamide is believed to stimulate respiration through stimulation of the carotid body (5). It has also been reported that vanillic diethylamide acts through stimulation of this receptor area (6); however, single and infused doses of vanillic diethylamide following bilateral denervation of the carotid body-sinus area produced similar effects to those seen prior to denervation. It would appear that the carotid body has little, if any, part in the mechanism of action of the compound.

The infusion of vanillic diethylamide into depressed cats decreased the depth of anesthesia in accordance with the rate of infusion. It would appear that this method of administration might lend itself well to clinical control of overdepressed patients, especially since vanillic diethylamide appears to be quickly metabolized.

The hypotensive effect of the compound produced by single intravenous injections into depressed animals was not observed following intra-arterial injection into the recipient animal in cross-circulation experiments. A hypertensive effect rather than the expected hypotensive response occurred in these animals. This might indicate that the hypotensive response was due to peripheral mechanisms rather than effects mediated through the central nervous system.

It appears that vanillic diethylamide is a potent central nervous system stimulant having primary effects on the medullary neural receptors with little or no effect taking place through the carotid body chemoreceptors or the carotid sinus receptors. It should also be noted that the cat appears to be extremely sensitive to the drug.

SUMMARY

1. The central stimulatory effects of vanillic diethylamide were studied in frogs, rats, dogs, and cats.

2. Vanillic diethylamide produced central stimulatory effects in all species tested.

3. The stimulatory action of vanillic diethylamide appears to be mainly due to direct stimulation of medullary receptor sites and not to stimulation of the carotid body-sinus complex.

4. Large doses of the compound produced transient hypotensive effects which could be eliminated by administering vanillic diethylamide by slow intravenous infusion.

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Hydrocholeresis in the Isolated Perfused Rat Liver

By W. W. TUTTLE, L. L. EISENBRANDT, and P. M. SCOTT

The construction and operation of a simplified isolated rat liver perfusion apparatus consisting of readily available components are described. In 30 determinations this procedure successfully maintained isolated rat liver function to a degree equivalent to that obtained by other investigators.

PIONEER STUDIES with perfused livers as reported by Bauer, *et al.* (1), were made by Mautner and Pick in 1915. These early perfusions, which employed saline perfusates, lead to the more recent and refined investigations of Brauer, *et al.* (2), who used expanded blood perfusates. This latter procedure required considerable glass manipulation, for the apparatus was tailored to the morphology of the liver. Young, *et al.* (3), later developed a rabbit liver perfusion apparatus which had the advantage of being composed largely of standard glassware, facilitating initial construction and replacement. An adaptation of this sytem was used in the work herein reported.

Isolated liver perfusion as a means of studying liver function and metabolism is useful because it permits hepatic activity to be studied in the absence of extrinsic nerve supply and lymphatic plexus. Brauer, *et al.* (2), reported that maintenance of a liver in near physiologic state was excellent if a blood-containing perfusate were used.

METHODS AND MATERIAL

Perfusion Apparatus.—The perfusion apparatus (Fig. 1) consisted of a perfusate reservoir, oxygenating column, organ chamber, circulating pump and filter system, hydrostatic perfusate reservoir, and a thermostatically (37.5°) controlled thermal plywood cabinet with a glass door. Heat was supplied by four 100-watt shielded light bulbs.

The reservoir [1] was a 100-ml. three-neck distilling flask with a standard taper 24/40 center-neck. All other ground-glass joints in this apparatus were standard taper 24/40. The oxygenation system consisted of an oxygenating column [2] made by fusing an outer and inner Pyrex joint to give a column of 20 cm. total length. The connecting tubes [3] on either end of this column were Pyrex with 105° side arms. The Pyrex inner joint [4], above the upper connecting tube, was cut to fit the lower neck of the organ chamber [5] with the aid of a bored-rubber stopper gasket. The organ chamber consisted of two 150-mm. desiccator covers with greased, ground-glass surfaces to insure an air-tight seal for good oxygen circulation through the apparatus. A small hole in the lower cover allowed for egress of the bile duct cannula. The organ mounting cradle [6] was made of No. 50-mesh stainless steel screen. Special attention is directed to the cannula attachment [7, 8] which was fashioned by fitting a rubber stopper in the upper desiccator neck with a short length of 9-mm. Pyrex glass tubing which extended above the stopper $^1/_4$ inch. An 11.5-cm. length of 7-mm. Pyrex tubing was slipped into the 9-mm. length, and the upper juncture of these sealed with a short length of rubber tubing. This allowed for sliding the inner glass tubing leading to the portal cannula up or down for positioning the excised liver. The double perfusion valve [11] was available from the Macalister-Bicknell Co., Cambridge, Mass. The blood filters [10] were Baxter Filterdrip assemblies which were modified to reduce their volume by use of 7-mm. Pyrex glass tubing extensions [13]. These extensions were inserted into the top of the assemblies and terminated a few millimeters above the filter elements. The double filter system enabled transfer of filters should one become clogged during a perfusion. The pulsating tube [12] was made from a 2-inch length of 22-mm. Pyrex glass tubing joined to a $1^1/_2$-inch length of 7-mm. Pyrex tubing. The diaphragm of this pulsating pump was a large rubber finger cot which was folded over the large end of the pulsating tube and held in place by rubber cement and closed by the rubber adapter cap of a disposable Baxter blood administration set. A pipetting machine [21] with valves removed was fitted with a lubricated 30-ml. syringe to provide the reciprocating action necessary to expand and collapse the finger cot diaphragm of the pulsating chamber. The hydrostatic reservoir [15] was made from a $2^1/_2$-inch length of 22-mm. Pyrex tubing attached at the bottom to a short length of 7-mm. Pyrex tubing. The overflow arm was made from 9-mm. Pyrex tubing with its lower end reduced to 7 mm. This side arm was attached to the reservoir 1 inch from the top. The increase in tube size in this arm permitted a noncontinuous overflow of the perfusate which bipassed the liver. This reservoir was elevated to give 21.5 cm. of hydrostatic pressure from overflow arm to portal vein of the liver. The infusion system [17] consisted of a Phipps and Bird syringe driver adapter fitted to a kymograph and adjusted to deliver 0.012 ml./min. of infusate into the system via the latex tubing above the organ chamber. A few drops of Dow Corning antifoam B were added to the saline humidifier [16] to prevent excessive foaming. The rubber connecting tubing used throughout this system was amber latex rubber tubing with 5 mm. i.d. and 1.5 mm. wall thickness. Especially constructed glass cannulas were used for the portal and vena cava cannulations, as adapted from the work of Werthessen (4).

The entire system except for the pulsating tube

Received April 28, 1961, from the University of Kansas City, School of Pharmacy, Kansas City, Mo.
Accepted for publication June 22, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

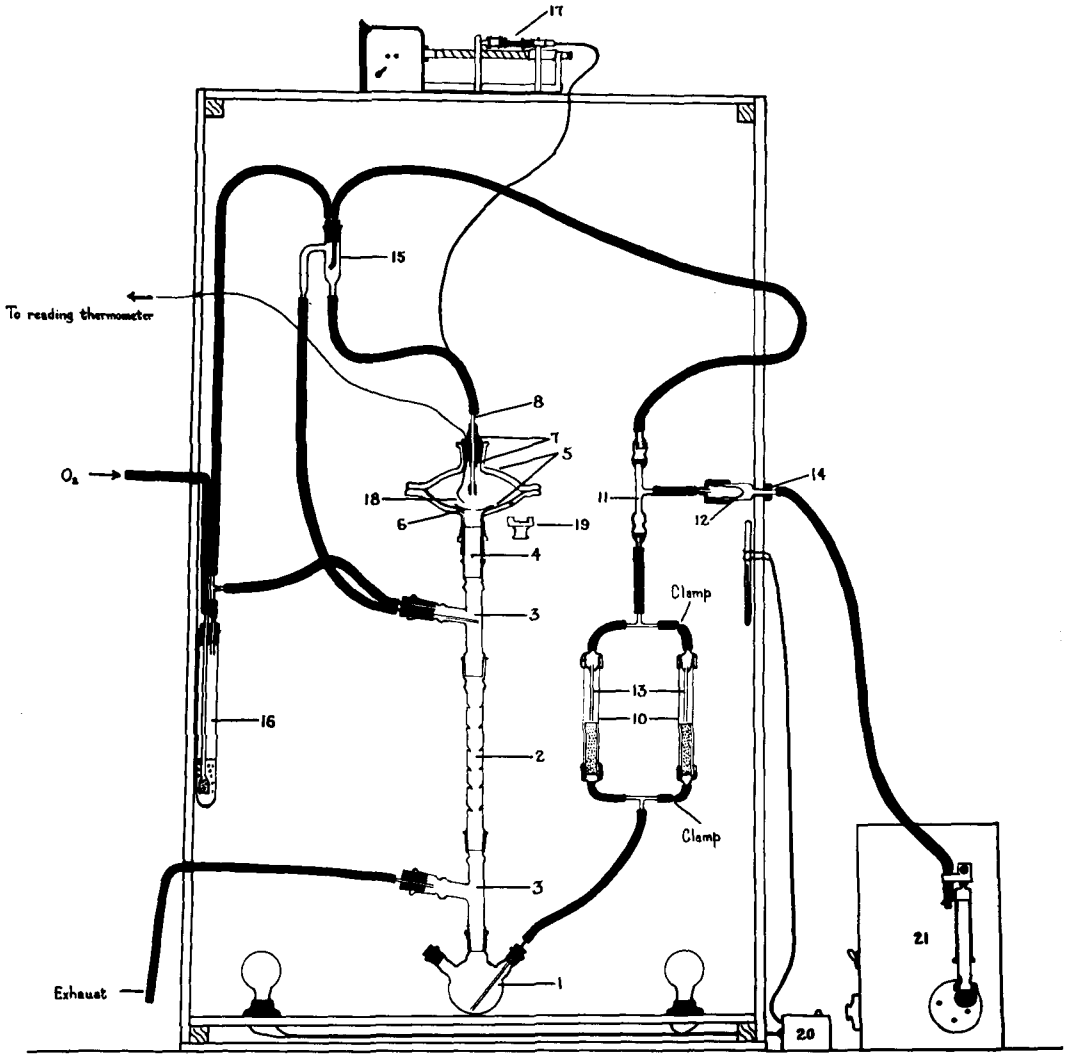


Fig. 1.—Liver perfusion apparatus. 1, Reservoir; 2, oxygenating column; 3, connecting tubes; 4, inner joint; 5, organ chamber; 6, organ mounting cradle; 7 and 8, adjustable portal cannula attachment; 10, dual blood filters; 11, double perfusion valve; 12, pulsating tube; 13, tubular extensions; 14, rubber friction washer; 15, hydrostatic reservoir; 16, gas saline humidifier; 17, infusion system; 18, thermometer thermocouple; 19, planchet magazine; 20, thermostat; 21, pipetting machine.

was steam sterilized prior to use, and the finger cot diaphragm of the pulsating tube was soaked with benzalkonium chloride 1:500 for 3 hours prior to assembly.

Perfusate.—The perfusate and infusate mixtures were adopted from Brauer, *et al.* (2), to give a basis for performance comparisons with his biliary flow rates. Perfusate solutions A (expanded physiologic saline)[1] and B (phosphate buffer system) were mixed, added to the reservoir and equilibrated with oxygen for 2 minutes, and then solution C (rat blood) was added. The infusate mixture consisted of an antibiotic and nutrient in an expanded saline medium.

Operative Technique.—Under ether anesthesia the bile duct of Sprague-Dawley rats was cannulated with P.E. 50 tubing. The inferior vena cava of the heparinized animal was ligated just superior to the right renal vein and divided. The inferior vena cava was cannulated just superior to the diaphragm and divided. The portal vein was cannulated last and severed. The organ was then removed by excising the diaphragm and dividing the suspensatory ligaments attached to the organ. The liver was mounted, diaphragm down, on the stainless steel cradle and attached to the filled perfusion system making sure all lobes were free for circulation. The bile duct cannula was placed through the hole in the organ chamber provided for it and positioned over the planchet magazine. Then the covers of the organ chamber were sealed. The thermocouple to the thermometer was then positioned to rest lightly on a lobe of the liver. Surface temperature varied from 38.5 to 39.5° which was within the temperature range of maximal bile production as reported by Brauer, *et al.* (5).

[1] The authors are indebted to the Armour Laboratories for the bovine plasma albumin used in the perfusate.

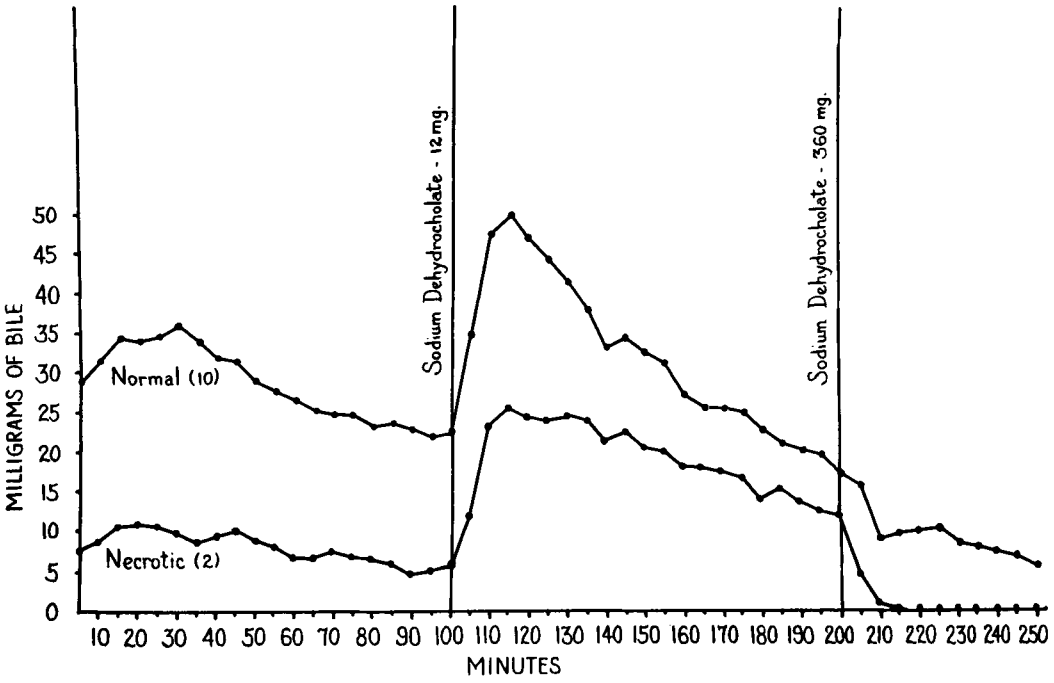


Fig. 2.—Bile flow of normal and necrotic isolated perfused rat livers.

Operation of Apparatus.—Oxygen flow was maintained at 2 L./min. The perfusate pumping rate was regulated to pass about one-half as much perfusate over the hydrostatic reservoir overflow as that which passed throuhg the organ. Perfusate flow varied somewhat in each individual organ, but the usual rate was 100 to 150 ml. per minute.

A properly functioning organ was evidenced by the gradual change in lobe color from dark to pink, as well as by the rate of bile flow. A nonperfusing lobe remained dark and required repositioning to initiate perfusion.

After an equilibration period of 15 minutes, bile was collected at 5-minute intervals in tared cup planchets and quickly weighed.

EXPERIMENTAL DATA

Fifteen Sprague-Dawley albino rats, ranging in weight from 350 to 400 Gm., served as liver donors in the early phases of the experiment where adjustment of the oxygenation rate was necessary, as well as the perfusion design and technique. In addition, 30 animals were used for varying periods of time. Of these, 12 were complete runs of 250 minutes and are included to illustrate the operation of the equipment. The high rate of failures indicates the technical complexities of isolated rat liver perfusion. Mean perfusion rates of 10 normal livers and two necrotic livers and their response to sodium dehydrocholate[2] are reported. The data are preliminary and are to be extended.

Twenty-four-hour liver necrosis was induced by an oral dose of 3 ml./Kg. of a 40% mixture of carbon tetrachloride in olive oil (6). Special care in handling these organs was needed because the carbon tetrachloride induced considerable fragility.

A rapid increase in bile flow occurred during the first 45 minutes of perfusion followed by a gradual decline for about the same time-interval, finally reaching a steady phase of flow of approximately 23 mg. per 5-minute interval (Fig. 2).

During the steady phase the initial 12-mg. dose of sodium dehydrocholate was given into the latex tubing of the hydrostatic reservoir overflow which allowed for maximal mixing with the perfusate before reaching the liver. The average bile flow (by weight) at the time of the dose was 22.2 mg., while the average peak bile flow 15 minutes later was 49.9 mg. per 5-minute interval, an increase of 125 ± 14.7%. During hydrocholeresis the perfusate flow was unchanged.

The initial high rate of bile flow was not as pronounced in the necrotic preparations but had the same general pattern as the normal. The necrotic livers exhibited considerably less biliary flow than their normal counterparts. The relative percentage response of the necrotic livers to the 12-mg. dose of sodium dehydrocholate was greater than in the normal livers. The average bile flow (by weight) of the necrotic livers at the time of the dose was 5.5 mg.; 15 minutes later the average peak bile flow was 25.3 mg. per 5-minute interval, a 360 ± 68.7% increase. Again the perfusate rate remained constant. The stimulated response was considerably more prolonged in the necrotic livers as compared to the normal.

After allowing 100 minutes for the initial response to sodium dehydrocholate (12 mg.) to subside, the 360-mg. dose was given as before. This dose was equivalent to the 200 mg./Kg. dose used routinely in this laboratory with *in situ* work.

[2] Appreciation is expressed to George A. Breon and Co. for the Dilabil-Sodium Injection.

The response from this massive dose resulted in an abrupt decrease in normal liver biliary flow from an average weight at the time of administration of 17.2 mg. to 5.9 mg. of bile per 5-minute interval at depth of depression, a 67.5 ± 11.8% decrease. Again the perfusate flow remained constant.

Although necrotic livers did not return to their stable biliary flow rate in 100 minutes, the 360-mg. dose of sodium dehydrocholate was given for experimental continuity. Bile flow decreased from an average flow at time of administration from 12.0 to 0.00 mg. of bile per 5-minute interval, a 100 ± 0.00% decrease. The perfusate flow remained constant as before. Obviously, the latter data are suggestive only.

DISCUSSION

The described liver perfusion unit differs from others in that standard glassware and simple cabinet construction were used. Using identical perfusate and infusate mixtures and the same hydrostatic pressure as Brauer, *et al.* (2), a normal bile flow pattern identical with his was found. However, the hydrocholeretic response was not proportionally as great as that from the 2 mg. of sodium dehydrocholate used by Brauer. Perhaps this was due to the addition of the drug by Brauer into the system just above the liver, which resulted in a greater initial hepatic concentration of drug.

The decrease in biliary flow due to the 360-mg. dose was apparently not a vascular response because the perfusate flow remained constant. During preliminary runs, large doses (360 and 840 mg.) of sodium dehydrocholate were found to elicit a depression of bile flow during the first hour of perfusion. Also, 2-mg. doses of sodium dehydrocholate given at 40, 95, 135, and 160 minutes resulted in equivalent increments in biliary flow indicating that the depressive response is a true reaction and not just the malfunction of a fatigued or subnormal organ. Goodman and Gilman (7) and Clark (8) noted that the toxic effects of bile salts resulted in inhibition of cholinesterase. Perhaps endogenously released acetylcholine caused a decrease in bile flow through biliary tract constriction.

The perfusion rate of the isolated perfused rat liver was considerably above the *in situ* rate of blood flow. Brauer, *et al.* (9), attributed this fivefold increase in flow rate to the inactivation of a hepatic vasoconstrictor factor resulting in full vasodilatation of the hepatic vascular tree.

The initial rise in bile flow may be due to a recovery from liver hypothermia following the surgical procedure. Brauer, *et al.* (10), noted that bile elaboration was depressed when liver temperature was reduced from its optimal value of about 40°. Upon rewarming, an excessive flow with increased bilirubin content resulted for a brief period. This surge was perhaps due to an accumulation of intermediate metabolites leading to excessive flow once metabolic balance was restored upon rewarming. The initial collections of bile in this work were noticeably darker, which quite possibly indicated an increment of bile salts.

It is quite apparent that necrotic liver data are insufficient for evaluation, but were included to indicate a trend.

SUMMARY

The assembly and operation of a functional isolated rat liver perfusion apparatus is described using simple materials and standard glassware. Preliminary data are included to illustrate the operation of the unit.

The mean per cent response of normal isolated perfused rat livers to 12 mg. of sodium dehydrocholate from point of administration to peak of hydrocholeretic rise was 125 ± 14.7% (22.2 to 49.9 mg. bile per 5-minute interval). The comparable response of necrotic livers was 360 ± 68.7% (5.5 mg. to 25.3 mg. bile).

The bile flow response to 360 mg. sodium dehydrocholate resulted in a decrease from point of administration to depth of depression of 67.5 ± 11.8% (17.2 to 5.9 mg. bile per 5-minute interval) for normal livers, and 100 ± 0.00% (12.0 to 0.00 mg. bile) for necrotic livers.

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Manometric Method for the Evaluation of Preservatives

By JOHN L. WAILES

A manometric method using the Warburg apparatus has been investigated as a possible screening technique for the evaluation of agents used as preservatives in pharmaceutical preparations. This procedure also was utilized to determine the relative effectiveness of different preservatives, and preservative-surfactant combinations. The respiratory effects on baker's yeast were determined with methylparaben, propylparaben, and sorbic acid, separately, and each in the presence of polysorbate 80. Each of the three preservatives caused a decrease in oxygen consumption by baker's yeast. In general, the order of effectiveness was propylparaben > sorbic acid > methylparaben. Polysorbate 80 decreased the effectiveness of each of the three preservatives. Sorbic acid was the least affected, except at a preservative concentration of 0.025%. These manometric results are rapidly obtained, informative, and in general agreement with results found by more time-consuming procedures.

VARIOUS METHODS such as the agar-cup-plate, agar streak, agar-disk-plate, and direct inoculation, with and without complex nutrients, have been used to test preservatives against a variety of organisms. These methods, though informative, are time consuming. Also, some question may arise as to the interaction of preservative with the complex nutrients (1, 2) and the different rates of diffusion of preservatives in these nutrients.

This investigation was undertaken to determine the feasibility of using a Warburg manometric procedure as a screening technique for the evaluation of agents used as preservatives in pharmaceutical preparations. The method was devised to reduce the time element and to employ a simple medium, thus obviating complex nutrients and the varying rates of preservative diffusion in a medium. Also, it was of interest to determine whether or not the method would reveal the relative effectiveness of different preservatives and preservative-surfactant combinations.

Sabalitschka and Boehm (3) were among the first investigators to use the *p*-hydroxybenzoate esters as antibacterial and antifungal agents. Since that time these esters and many other substances have been investigated, compared, and recommended for their preservative activity (4–12). The evidence in the literature is abundant concerning the interaction between methyl- and propylparaben with nonionic surfactants (13–20).

The use of sorbic acid as a pharmaceutical preservative is relatively new compared with the parabens. Cosgrove, *et al.* (21), and later deNavarre (22) were among the first investigators to employ sorbic acid as a preservative in pharmaceuticals. In addition, deNavarre (19, 23), Barr and Tice (18), Charles and Carter (24), and recently Blaug and Ahsan (25) have investigated the effectiveness of sorbic acid in the presence of nonionic surfactants.

EXPERIMENTAL

The direct method of Warburg (26) was used to measure the amount of oxygen consumed by the yeast cells. The respiratory effects on baker's yeast were determined with methylparaben, propylparaben, and sorbic acid. Each preservative was tested separately with five different w/v concentrations: 0.025, 0.05, 0.1, 0.2, and 0.4%. In addition, each concentration of preservative was combined with five separate w/v concentrations of polysorbate 80:[1] 0.25, 0.5, 1.0, 2.0, and 5.0%. The oxygen uptake by the yeast cells in the presence of the preservative and the preservative-surfactant combination was compared with the control over a period of 90 minutes.

Each Warburg reaction flask contained a final volume of 3.0 ml. The center well of each flask contained 0.2 ml. of 20% potassium hydroxide in which a small, pleated, filter paper was inserted. The side arm contained 0.2 ml. of yeast, equivalent to 1.25 mg. of dried baker's yeast, suspended in sterile 0.05 *M* phosphate buffer (27) pH 6.0. The yeast suspension was prepared by adding 100 ml. of the sterile phosphate buffer to 625 mg. of dried yeast. The suspension was stirred for 30 minutes prior to pipetting to ensure an even dispersion of the yeast cells.

The main compartment of the flask contained 2.6 ml. of the preservative dissolved in an alcohol-phosphate buffer-dextrose solution. As an example, in order to prepare a 0.1% solution of the preservative, 107.7 mg. of the preservative was dissolved in 3.4 ml. of 95% ethyl alcohol and diluted to 100 ml. with sterile 0.05 *M* phosphate buffer, pH 6.0,

[1] Tween 80, Atlas Powder Co., Wilmington, Del.

Received April 28, 1961, from the School of Pharmacy, Montana State University, Missoula.
Accepted for publication June 22, 1961.
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This investigation was supported in part by a research grant, E-2788, from The National Institute of Allergy and Infectious Diseases, Public Health Service.

containing 5% dextrose. When 2.6 ml. of this solution was diluted with 0.2 ml. of the yeast suspension contained in the side arm, the resulting solution consisted of 0.1% of the preservative and 3.0% ethyl alcohol. The control flasks contained 2.6 ml. of the alcohol-phosphate buffer-dextrose solution in the main compartment in addition to the 0.2 ml. yeast suspension in the side arm, and the potassium hydroxide in the center well.

The flasks were placed in the constant temperature water bath. All tests were conducted at 37 ± 0.01°. The Warburg was adjusted to 120 cycles per minute with a 3-inch stroke. After a 10 minute equilibration period, the contents of the side arm were tipped into the main compartment of the reaction flasks. Manometer readings were recorded every 10 minutes for a 90-minute period.

At the end of 90 minutes, one standard loopful was transferred from the main compartment of each flask to 20 ml. of sterile Sabouraud maltose broth. These tubes were incubated at 37° for 18 hours, and then observed to determine either the absence or presence of growth. Those tubes that showed no growth were incubated for a total of 72 hours, and again observed.

All solutions and the yeast suspension were prepared immediately prior to use. Each concentration of preservative and preservative-surfactant combination was run in triplicate and each experiment repeated three times.

RESULTS AND DISCUSSION

It was considered necessary to determine the respiratory effects of each component of the substrate. Table I reveals that baker's yeast in phosphate buffer consumed very little oxygen over the 90-minute time period, thus showing little endogenous respiration. However, approximately a ninefold increase in oxygen consumption was observed with the addition of 5% w/v dextrose. Oxygen consumption was further increased by the addition of alcohol. The increase was observed with the subsequent addition of 1, 3, and 5% (v/v) alcohol. Therefore, alcohol at any one of these concentrations could be used as a solvent for the preservatives without any inhibition of respiration caused by the presence of alcohol. Three per cent alcohol was arbitrarily chosen as the solvent in this investigation.

It was observed that a 10% concentration of alcohol caused a decrease in oxygen consumption. Therefore, a 10% concentration is in itself inhibiting to yeast respiration. This inhibition was more pronounced at 15%, and complete inhibition was noted with 20% alcohol.

When polysorbate 80 was added to the phosphate buffer-5% dextrose-3% alcohol substrate, no significant change in oxygen consumption was observed during the 90-minute period. Barr and Tice (18), although using different organisms, concluded that microorganisms produce esterases that split the fatty acid from the surfactant. They also stated that the possibility of the microorganisms utilizing the surfactants for nutritive purposes was remote. The insignificant change in oxygen consumption by yeast cells in the presence of polysorbate 80 tends to confirm their work.

Table II reveals that propylparaben is superior to methylparaben in inhibiting the oxygen consumption of baker's yeast. These manometric results are in accordance with those obtained by Sabalitschka (5). Also, it may be seen that sorbic acid has a greater inhibitory effect than methylparaben, but not as great as propylparaben in the dextrose-alcohol substrate buffered at a pH of 6.0. These results are in agreement with those reported by Osman and El-Mariah (28). Methylparaben is superior to sorbic acid at the lowest preservative concentration of 0.025%. This exception was also observed when the preservatives were tested in the presence of polysorbate 80 (Tables III–V).

A comparison of the results found in Tables III–V shows that an increase in the percentage of polysorbate 80 causes a decrease in the effectiveness of methylparaben, propylparaben, and sorbic acid in inhibiting the respiration of the yeast cells.

Although sorbic acid is the least affected by the surfactant, propylparaben is superior in the presence of small quantities (0.25 and 0.5%) of polysorbate 80. Thus, in pharmaceutical preparations containing less than 1% polysorbate 80, propylparaben would be the best preservative for inhibiting the growth of yeast. In instances where the percentage of surfactant is greater than 1%, sorbic acid would be more effective. These results support the findings of Charles and Carter (24) in that sorbic acid in the presence of nonionic surfactants provided protection of cosmetic formulations against contamination by various organisms.

Experiments also were undertaken in an attempt to correlate either the killing or the inhibiting action with the per cent of respiratory inhibition. At the end of the 90-minute period, one standard loopful from the main compartment of each flask was transferred to a tube containing 20 ml. of sterile Sabouraud dextrose broth. The tubes were incubated at 37° for a period of 18 hours and then observed for growth. If no growth was apparent, the tubes were incubated for a total of 72 hours, and observed again for growth.

No growth appeared at the end of 72 hours in the tubes containing inoculum from the flasks that showed an inhibition of 95% or greater. Those preservatives that produced this degree of inhibition evidently killed the yeast cells within this 90-

TABLE I.—MICROLITERS OF OXYGEN CONSUMED BY 1.25 MG. OF BAKER'S YEAST IN VARIOUS SUBSTRATES

Substrate	Av. μl. O_2 Consumed in 90 min.
Phosphate buffer pH 6.0	16
5% Dextrose[a] in phosphate buffer pH 6.0	143
5% Dextrose + 1% alcohol[b] in phosphate buffer pH 6.0	162
5% Dextrose + 3% alcohol in phosphate buffer pH 6.0	186
5% Dextrose + 5% alcohol in phosphate buffer pH 6.0	217
5% Dextrose + 10% alcohol in phosphate buffer pH 6.0	140
5% Dextrose + 15% alcohol in phosphate buffer pH 6.0	56
5% Dextrose + 20% alcohol in phosphate buffer pH 6.0	0

[a] w/v Solution. [b] v/v Solution.

TABLE II.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY METHYLPARABEN, PROPYLPARABEN, AND SORBIC ACID IN 90 MINUTES

Concentration of Preservative, % w/v	Per Cent Inhibition		
	Methylparaben	Propylparaben	Sorbic Acid
0.4	92 ± 0.632[a]	100 ± 0.000	96 ± 3.346
0.2	43 ± 1.549	99 ± 1.000	83 ± 0.812
0.1	33 ± 2.024	99 ± 1.000	65 ± 1.483
0.05	26 ± 1.048	90 ± 1.000	30 ± 1.500
0.025	18 ± 1.483	58 ± 0.707	6 ± 0.812

[a] Standard deviation.

TABLE III.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY METHYLPARABEN AND METHYLPARABEN-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Methylparaben, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	92 ± 0.632[a]	81 ± 1.897	75 ± 2.121	63 ± 1.581	48 ± 1.897	30 ± 2.121
0.2	43 ± 1.549	43 ± 2.121	39 ± 1.000	32 ± 1.581	26 ± 0.000	19 ± 1.581
0.1	33 ± 2.024	25 ± 1.732	22 ± 1.224	21 ± 2.121	16 ± 1.000	9 ± 1.000
0.05	26 ± 1.048	19 ± 1.000	18 ± 1.000	15 ± 0.707	7 ± 1.000	0 ± 0.000
0.025	18 ± 1.483	8 ± 0.707	7 ± 1.000	5 ± 0.000	0 ± 0.000	0 ± 0.000

[a] Standard deviation.

TABLE IV.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY PROPYLPARABEN AND PROPYLPARABEN-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Propylparaben, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	100 ± 0.000[a]	99 ± 1.000	98 ± 1.000	98 ± 1.000	82 ± 0.707	55 ± 0.707
0.2	99 ± 1.000	98 ± 0.000	96 ± 1.224	75 ± 1.000	61 ± 2.073	38 ± 0.707
0.1	99 ± 1.000	88 ± 0.707	64 ± 1.581	58 ± 1.788	47 ± 1.581	20 ± 2.144
0.05	90 ± 1.000	58 ± 1.224	55 ± 0.707	42 ± 1.732	24 ± 1.000	5 ± 0.000
0.025	58 ± 0.707	46 ± 1.000	36 ± 1.732	24 ± 2.236	12 ± 1.303	0 ± 0.000

[a] Standard deviation.

TABLE V.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY SORBIC ACID AND SORBIC ACID-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Sorbic Acid, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	96 ± 3.346[a]	98 ± 0.707	96 ± 1.581	98 ± 1.224	90 ± 0.707	87 ± 1.000
0.2	83 ± 0.812	83 ± 0.707	81 ± 1.732	81 ± 1.000	78 ± 1.581	64 ± 2.345
0.1	65 ± 1.483	64 ± 2.345	61 ± 2.121	62 ± 1.224	59 ± 1.000	49 ± 3.240
0.05	30 ± 1.500	26 ± 1.581	34 ± 0.707	32 ± 2.549	34 ± 1.224	18 ± 1.288
0.025	6 ± 0.812	0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000

[a] Standard deviation.

minute period. This could be considered as self-sterilization.

In those experiments where the percentage of inhibition ranged from 86 to 92%, growth appeared in the tubes at the end of 72 hours, but not at 18 hours. This showed that there were some yeast cells living and able to reproduce in a favorable environment. Had the time limit been extended for more than 90 minutes, it is possible that those few remaining cells might have been killed.

All other tubes from the remaining experiments showed growth at the end of the 18 hours of incubation. Therefore, those results represent merely a degree of inhibition, but not one of self-sterilization.

CONCLUSIONS

1. A manometric method has been used to determine the relative effectiveness of preservatives, and preservative-surfactant combinations on the respiration of baker's yeast.

2. Propylparaben was superior to either sorbic acid or methylparaben in inhibiting yeast respiration in a substrate composed of 5% dextrose, 3% alcohol, and buffered at a pH of 6.0.

3. The activity of sorbic acid was the least affected in the presence of polysorbate 80.

4. In the presence of small quantities (0.25 and 0.5%) of polysorbate 80, propylparaben showed the greatest inhibitory powers. However in greater concentrations of the surfactant (1, 2, and 5%) sorbic acid was more effective.

5. Methylparaben was the least effective of the compounds tested, alone or in the presence of polysorbate 80.

6. On the basis of these manometric results using baker's yeast as the test organism, further investigation is considered justified using this technique with other organisms, preservatives, and surfactants.

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Antiradiation Compounds I

Acylated Derivatives of β-Mercaptoethylamine

By WILLIAM O. FOYE, RONALD N. DUVALL, and JAMES MICKLES

A series of S-acyl derivatives of β-mercaptoethylamine (MEA) has been prepared in an attempt to provide compounds having protective effects in animal cells against ionizing radiation and which are less toxic than MEA itself. In general, toxicity to animals was reduced and good protective ability against X-radiation was retained in some of the esters. The dithiocarbamic acid of MEA was also prepared, but attempts to acylate this compound, which showed good antiradiation effects in close to toxic dose levels in mice, gave a variety of decomposition products.

Beta-Mercaptoethylamine (cysteamine, MEA) has been reported one of the most effective compounds discovered for protecting animal cells against the deleterious effects of ionizing radiation (1). This compound is somewhat toxic for human use (2), however, so an attempt has been made to mask the toxic effects by preparing derivatives subject to hydrolysis *in vivo* which would afford a liberation of MEA over a period of time. Several acylating agents were selected for this purpose.

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The authors express their gratitude for this support as well as for the testing and toxicity data supplied by Dr. D. P. Jacobus.

The hydrochloride of MEA was utilized on the assumption that acylation of the nitrogen would be prevented. Conventional blocking agents would be difficult to remove in the presence of the thioester formed, and nonhydrolyzable substituents on the nitrogen have generally resulted in diminution or loss of radio-protective action. Attempts to prepare the S-carbobenzoxy derivative of MEA, furthermore, resulted in the formation of S-benzylmercaptoethylamine. Also, MEA hydrochloride failed to react with ethyl chloroformate. However, the use of the hydrochloride of MEA was successful in preventing formation of N-acyl derivatives.

Some difficulty was experienced in finding a suitable solvent for both MEA·HCl and the selected acyl chlorides, but the reaction was found to take place in either dimethylformamide or an

4. In the presence of small quantities (0.25 and 0.5%) of polysorbate 80, propylparaben showed the greatest inhibitory powers. However in greater concentrations of the surfactant (1, 2, and 5%) sorbic acid was more effective.

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Antiradiation Compounds I

Acylated Derivatives of β-Mercaptoethylamine

By WILLIAM O. FOYE, RONALD N. DUVALL, and JAMES MICKLES

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Some difficulty was experienced in finding a suitable solvent for both MEA·HCl and the selected acyl chlorides, but the reaction was found to take place in either dimethylformamide or an

TABLE I.—ESTERS OF 2-MERCAPTOETHYLAMINE HYDROCHLORIDE

$$R{-}C(=O){-}S{-}CH_2{-}CH_2{-}NH_2\cdot HCl$$

R	M.p., °C.	Purification Solvent	Reaction Time, hr.	Yield, %	Formula	Analyses Calcd.	Found
CH_3	130–132	Benzene-ether	1/2	84	$C_4H_{10}ClNOS$	S: 20.6	21.4
C_3H_7	86–88	Benzene-ether	1/2	55	$C_6H_{14}ClNOS$	S: 17.4	17.7
C_4H_9	98–100	Benzene-ether	1/2	74	$C_7H_{16}ClNOS$	S: 16.2	16.3
C_5H_{11}	108–110	Benzene-ether	1/2	75	$C_8H_{18}ClNOS$	S: 15.1	15.7
C_6H_{13}	106–108	Benzene-ether	1/2	86	$C_9H_{20}ClNOS$	S: 14.2	14.6
C_7H_{15}	111–113	Absolute ethanol	2	85	$C_{10}H_{22}ClNOS$	S: 13.4	13.9
$C_{13}H_{27}$	126–128	Water	2	75	$C_{16}H_{34}ClNOS$	C: 59.2	59.3
						H: 10.5	10.7
						S: 9.9	10.2
CH_2Cl	126–128	Benzene-ether	1/2	85	$C_4H_9Cl_2NOS$	C: 25.3	25.3
						H: 4.8	4.7
						S: 16.9	16.7
C_6H_5	170–172	Absolute ethanol	2	82	$C_9H_{12}ClNOS$	S: 14.7	15.1

TABLE II.—ANTIRADIATION PROPERTIES OF MEA DERIVATIVES IN MICE[a]

Compound	Drug Level, mg./Kg.[b]	Dose, r	30-Day Survival, % Treated	Controls
S-Acetyl-2-mercaptoethylamine	400	800	60	0
	400	575	80	20
S-Chloroacetyl-2-mercaptoethylamine	600	800	0	0
	600	575	85	70
S-Octanoyl-2-mercaptoethylamine	600	800	60	0
	600	575	90	50
S-Myristoyl-2-mercaptoethylamine	400	800	0	0
	400	575	65	35
S-Benzoyl-2-mercaptoethylamine	225	800	10	0
	225	575	85	75
2-Mercaptoethyldithiocarbamic acid	350	800	75	0
	350	575	95	40

[a] Determined at the Walter Reed Army Institute of Research by D. P. Jacobus. [b] Administered 15 minutes prior to X-irradiation.

excess of the acyl chloride. Physical properties and reaction data for the esters prepared are listed in Table I.

Since dithiocarbamates have also shown relatively good protective effects against ionizing radiation, the dithiocarbamic acid of MEA was prepared, which may be considered an acylation with carbon disulfide. A two-phase system of carbon disulfide and ammonia water was applicable for this preparation; apparently the ammonium salt of the dithiocarbamic acid of MEA, which would be expected, was hydrolyzed under the reaction conditions, and the free acid was obtained.

Relatively good antiradiation properties in mice were found with the dithiocarbamic acid of MEA (see Table II), but the compound was found to be unstable in air, and close to toxic dosage levels were necessary for protection against lethal doses of X-radiation. Accordingly, attempts were made to acylate the β-mercapto group of this compound with the intention of increasing stability and reducing toxicity. Reaction of MEA dithiocarbamic acid with acetyl chloride at various temperatures, however, resulted in decomposition with the loss of hydrogen sulfide or carbon disulfide. Three products were identified from the attempted acetylation: 2-mercaptothiazoline, S-acetyl-β-mercaptoethylamine hydrochloride, and β-mercaptoethylamine disulfide dihydrochloride. See I. Attempted acylation in buffered aqueous solution also resulted in decomposition of the dithiocarbamic acid. The high degree of insolubility of this compound in organic media proved to be a major handicap.

The reverse procedure of dithiocarbamation of an acyl derivative of MEA was also investigated. The reaction of S-benzoyl-β-mercaptoethylamine hydrochloride with carbon disulfide and ammonia water was accordingly carried out, but the major product proved to be N-benzoyl-β-mercaptoethylamine. The migration of the benzoyl group appeared to be nearly instantaneous, as shown by immediate ether extraction of a solution of S-benzoyl-β-mercaptoethylamine hydrochloride when made basic with ammonia water. Similar results were obtained when the S-acetyl, S-octanoyl, and S-myristoyl esters of MEA were made alkaline in ammonia water; no evidence of dithiocarbamation was found. See II. The desired reaction was therefore found to be im-

possible in either neutral, acid, or basic aqueous media. Similar acyl migrations from sulfur to nitrogen have been observed by others (3).

Reaction of the dithiocarbamic acid of MEA with acid chlorides in ether also gave products that decomposed either on storage in a desiccator or on suspension in water. The N-acyl derivatives were isolated from this reaction, however. Use of pyridine and acetic anhydride likewise resulted in the formation of N-acetyl-2-mercaptoethylamine, a known compound.

Antiradiation Properties.—Tests for the ability of these compounds to protect mice against X-radiation were carried out at the Walter Reed Army Institute of Research under the direction of Dr. D. P. Jacobus. The S-acetyl derivative of MEA was found to provide 60% protection over a 30-day survival period when the mice were irradiated at a dose of 800 r. An 80% survival was observed after a dose of 575 r, nontoxic doses of compound being injected in each case 15 minutes prior to exposure. The S-octanoyl ester gave similar results. The S-myristoyl ester provided 65% protection against a dose of 575 r, but was ineffective against 800 r. The dithiocarbamic acid gave 75% protection against 800 r, and 95% protection against 575 r, making this compound almost the equal of MEA itself. These results are summarized in Table II.

The benzoyl ester of MEA was not appreciably effective, affording only 10% protection from 800 r. The chloroacetyl ester, however, which would be expected to hydrolyze in water at a somewhat more rapid rate than the other esters tested (4), surprisingly gave no protection at all. It is possible that the compound underwent polymerization or cyclization before reaching the cells, when neutralized prior to injection.

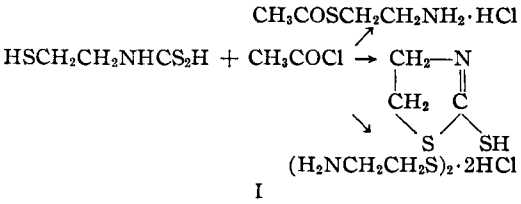
The acute intraperitoneal toxicities in mice of several of the esters are recorded in Table III, where they may be compared to the toxicity of 2-mercaptoethylamine. It is apparent that a lowering of toxicity has resulted on ester formation. Although hydrochlorides of the esters were used, a valid comparison may be made with 2-mercaptoethylamine itself, since neutralization would be expected to occur fairly rapidly *in vivo*.

On the basis of the mixed disulfide hypothesis (5) of radiation protection by mercaptans, the S-acyl derivatives of MEA would be expected to suffer hydrolysis *in vivo*, thus liberating MEA to form mixed disulfides with vital thiol groups in the cells. However, rearrangement to the N-acyl derivatives may take place, which is shown here to occur quite readily at pH's above 7. This, too, would provide a free thiol group for mixed disulfide formation, but the basicity of the nitrogen, usually present in compounds possessing antiradiation ability, would be lost. Antiradiation tests on the N-acyl derivatives should help resolve the question of the necessity of a basic nitrogen in compounds of this type.

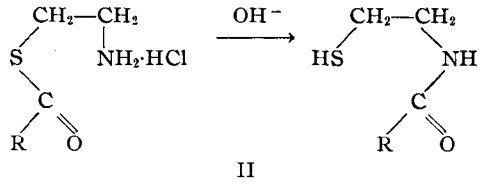
TABLE III.—INTRAPERITONEAL TOXICITIES OF MEA DERIVATIVES IN MICE[a]

Compound	Dose in Mice, mg./Kg.	Mortality, Acute	Dead/Total 10 Days
2-Mercaptoethylamine	230[b]	..	..
	350[c]	..	..
S-Acetyl-2-mercaptoethylamine·HCl	400	0/5	0/5
	500	2/5	4/5
S-Chloroacetyl-2-mercaptoethylamine·HCl	800	0/5	0/5
	1000	0/5	4/5
S-Octanoyl-2-mercaptoethylamine·HCl	650	0/5	0/5
	800	1/5	2/5
S-Myristoyl-2-mercaptoethylamine·HCl	400	0/5	0/5
	800	0/5	0/5
S-Benzoyl-2-mercaptoethylamine·HCl	250	0/5	0/5
	350	2/3	4/5
2-Mercaptoethyldithiocarbamic acid	400	0/5	0/5
	500	0/5	1/5
	600	4/5	5/5

[a] Determined at the Walter Reed Army Institute of Research by D. P. Jacobus. [b] LD_{50} value in rats obtained by Bonati, F., *Arch. ital. sci. farmacol.*, **9**, 125(1959). [c] LD_{50} value in mice obtained by Bacq, Z. M., and Herve, A., *Schweiz. med. Wochschr.*, **82**, 1018(1952).



I



II

EXPERIMENTAL

Melting points were taken on a Fisher-Johns block and are uncorrected. Carbon-hydrogen analyses were done by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass.

2-Mercaptoethylamine Hydrochloride.—A modification of the procedure of Mills and Bogert (6) was used. Ethyleneimine (27.5 ml., 0.52 mole) dissolved in 205 ml. of absolute ethanol was added dropwise, with stirring, during 1 hour to 200 ml. of absolute ethanol. A stream of dry hydrogen sulfide was passed into the ice-cooled solution simultaneously. The resulting solution was partially dis-

tilled under nitrogen and reduced pressure, and dry hydrogen chloride was then bubbled through the remaining solution until it was acidic. The solvent was distilled under nitrogen and reduced pressure, and the resulting oil was allowed to crystallize by being chilled in tightly stoppered bottles. A 72% yield was obtained of material melting at 70°, which agrees with the reported value (7).

Esters of 2-Mercaptoethylamine Hydrochloride.—The following procedure is representative. A mixture of 2-mercaptoethylamine hydrochloride (4.5 Gm., 0.04 mole) and myristoyl chloride (15 ml., Eastman Organic Chemicals) was refluxed on a water bath for 2 hours or until the mixture solidified (the lower esters, with the exception of the benzoyl, gave solutions). The solid mass (which generally resulted on cooling) was pulverized, washed with benzene and ether, and recrystallized from absolute ethanol. Washing with water was possible in the case of the myristoyl ester. A yield of 75% was obtained of material melting at 126–128°.

Anal.—Calcd. for $C_{16}H_{34}ClNOS$: C, 59.2; H, 10.5; S, 9.9. Found: C, 59.3; H, 10.7; S, 10.2.

Reaction of 2-Mercaptoethylamine with Benzyloxycarbonyl Chloride.—2-Mercaptoethylamine hydrochloride (10.0 Gm., 0.09 mole) was refluxed with 25 ml. of benzyloxycarbonyl chloride for 20 hours on a water bath or 4 hours at 150°, when a clear solution resulted. Benzene was added, and an oil separated which soon crystallized. The product was recrystallized from absolute alcohol with the addition of a large amount of ether. The product, 2-benzylthioethylamine hydrochloride, which did not give a mercapto test with iodine, melted at 112–114° [Walton (8) reported 120–136°].

Anal.—Calcd. for $C_9H_{14}ClNS$: C, 53.1; H, 6.9; S, 15.7. Found: C, 52.6; H, 6.7; S, 15.7.

2-Mercaptoethyldithiocarbamic Acid.—A solution of 2-mercaptoethylamine hydrochloride (11.3 Gm., 0.1 mole) in 20 ml. of water was added dropwise, with stirring, to an ice-cooled mixture of 7 ml. of carbon disulfide (0.1 mole) and 22 ml. of ammonia water (0.32 mole). Stirring was continued for an hour after the addition was complete, and the yellow product was isolated and dried rapidly on blotting paper at room temperature. The product was stored in a desiccator under nitrogen at refrigerator temperature. The yield was 10 Gm. (65%) of material melting at 76–78°.

Anal.—Calcd. for $C_3H_7NS_2$: C, 23.5; H, 4.6; S, 62.7. Found: C, 23.7; H, 4.9; S, 61.9.

Acetylation of 2-Mercaptoethyldithiocarbamic Acid.—2-Mercaptoethyldithiocarbamic acid (2 Gm., 0.013 mole) was treated with excess acetyl chloride both at room temperature and at 0–5° and allowed to stand for a half-hour. Hydrogen sulfide was liberated and an oil was formed which was washed thoroughly with benzene and ether. The oil was dissolved in alcohol and pale yellow crystals appeared. The material melted at 127–130° and was identified as S-acetyl-2-mercaptoethylamine hydrochloride [lit. (9) m.p. 137°].

Anal.—Calcd. for $C_4H_{10}ClNOS$: S, 20.6. Found: S, 21.4.

A water-insoluble product that appeared in the original reaction melted at 101–103° and was identified as 2-mercaptothiazoline [lit. (10) m.p. 101–102°].

A small amount of material melting at 210° was also isolated from the alcohol extract of the oil. This proved to be 2-mercaptoethylamine disulfide dihydrochloride [lit. (6) m.p. 212–212.5°].

Acylation of 2-Mercaptoethyldithiocarbamic Acid in Ether.—Freshly prepared 2-mercaptoethyldithiocarbamic acid (2.0 Gm., 0.013 mole) was suspended in ether (100 ml.) surrounded by an ice pack. An excess of octanoyl chloride was added dropwise, and the yellow material turned to an orange color in a few minutes. The orange product was filtered and washed thoroughly with ether. As soon as the ether evaporated, however, the crystals turned yellow and became oily, which took place even on storage in a desiccator. After several days in a desiccator, the yellow product was dry and proved to be a mixture of N-octanoyl-2-mercaptoethylamine, m.p. 46–48°, and bis(2-aminoethyl) disulfide dihydrochloride, m.p. 209–210°.

N-Octanoyl-2-mercaptoethylamine.—S-Octanoyl-2-mercaptoethylamine hydrochloride (5.0 Gm., 0.02 mole) was dissolved in 50 ml. of water. The solution was made alkaline with ammonia water and an oily precipitate formed immediately. The mixture was extracted with two 100-ml. portions of ether, the extract was dried over sodium sulfate, and the ether was removed under a current of nitrogen. The gray-white product was recrystallized from 25% aqueous ethanol and 3.5 Gm. (82%) of material was obtained which melted at 46–48°. It decolorized iodine solution rapidly.

Anal.—Calcd. for $C_{10}H_{21}NOS$: C, 59.1; H, 10.3; S, 15.7. Found: C, 59.7; H, 9.9; S, 15.1.

N-Myristoyl-2-mercaptoethylamine.—This compound was prepared in the same manner as the N-octanoyl derivative.

Anal.—Calcd. for $C_{16}H_{33}NOS$: C, 66.9; H, 11.5. Found: C, 67.5; H, 11.4.

Attempted Dithiocarbamation of S-Acyl Esters of 2-Mercaptoethylamine Hydrochloride.—An ice-cooled aqueous solution of S-benzoyl-2-mercaptoethylamine hydrochloride (2.0 Gm.) was made basic with concentrated ammonia water and an equivalent of carbon disulfide was added dropwise with stirring. The yellow solution was made very slightly acidic with hydrochloric acid and extracted with ether. The ether extract was evaporated under a current of nitrogen and a colorless product crystallized, m.p. 65–67°. The product reduced iodine solution rapidly and was shown to be N-benzoyl-2-mercaptoethylamine [lit. (11) m.p. 69–71°].

Anal.—Calcd. for $C_9H_{11}NOS$: S, 17.7. Found: S, 17.8.

An identical product was obtained when an aqueous solution of S-benzoyl-2-mercaptoethylamine hydrochloride was made basic with ammonia water and extracted with ether.

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Kinetics of Degradation in Solution of Epinephrine by Molecular Oxygen

By THEODORE D. SOKOLOSKI† and TAKERU HIGUCHI

The rate of degradation of epinephrine in aqueous solution at pH 5 by molecular oxygen has been shown to be extremely complex functions of both the amine and oxygen concentrations. The kinetic studies, based on chromatographic isolation of epinephrine as its triacetate, indicate that at the pH used (*a*) the degradation products had no detectable effect on the observed oxidative rate, (*b*) the dependency on the catecholamine ranged from 1.0 to 0.5 depending on oxygen tension, and (*c*) conversely, the dependency on oxygen varied from over 1 to less than 0.4 depending on epinephrine concentration.

THE MECHANISMS and kinetics of the reaction between molecular oxygen and epinephrine have presented a challenge to pharmaceutical workers ever since the isolation of the hormone in 1901 by Takamine. Despite the long history of effort made in these directions, however, only a few reliable basic landmarks have been established concerning the reaction system because of (*a*) the lack of a suitable analytical procedure for the intact catecholamine and (*b*) the complexity of the oxidative process involved. Such paramountly important information as the rate dependencies of the concentration of the amine and oxygen had not been previously determined. In the present report the results of an investigation into these basic relations are given.

The influence of various factors on the rate of loss of epinephrine from its solutions has been attempted previously by numerous workers using a number of different assay techniques. Berry and West (1) have investigated the stability of epinephrine at different hydrogen ion concentrations by using a bioassay based on the response of a frog's heart to the drug. Notable among those methods based on colorimetric determinations was that of Mazur, Green, and Shorr (2) who studied the effect of drug concentration. Probably the most widely used method has been one based on some oxygen uptake technique. Wang (3), Trautner and Bradley (4), and Chaix, Chauvet, and Jezequel (5) have offered contributions based on this method of study. In none of the earlier investigations, unfortunately, was the rate of disappearance of the catecholamine itself observed. Since the oxygen uptake technique measures gas consumption, not only by epinephrine but also by a number of its breakdown products which are even stronger reducing agents, results obtained cannot be directly related to the primary reaction. Bioassay methods when used to follow epinephrine concentration also suffer from the fact that oxidation products can interfere with the determination. Ruiz-Gijon (6) has pointed out that adrenalinequinone, an oxidation product of epinephrine, has the same physiological properties as epinephrine. The results of any method based on a colorimetric determination should properly apply only to the catechol chromophore or the presence of phenolic hydroxyl groups.

The present investigation offers, for the first time, experimental results concerning the rate dependencies on the concentration of epinephrine and oxygen, based on a method which follows the loss of the drug by a direct and reproducible analysis of the intact molecule. The procedure was based on a modification of the U.S.P. XV method which successfully determines epinephrine in the presence of its oxidation products (7). The advantages that such a method for determining drug concentration holds over those techniques used by earlier workers is obvious.

EXPERIMENTAL

The dependency of the rate of autoxidation of epinephrine with respect to drug and oxygen concentrations was studied by reacting solutions of the amine at pH 5.0, selected for a convenient rate of degradation, and an ionic strength of 0.5 μ under various oxygen pressures at 65°.

Solutions of epinephrine at concentrations between 5×10^{-3} M and 0.2 M were prepared by weighing amounts of epinephrine base,[1] reported by the manufacturer and found on analysis to be more than 98% pure, into 0.05 M acetate buffer. The pH was adjusted with acetic acid and the ionic strength brought to the desired value by adding calculated amounts of potassium chloride. All

Received April 20, 1961, from the School of Pharmacy, University of Wisconsin, Madison.

Accepted for publication May 2, 1961.

This paper was taken in part from a thesis submitted by T. D. Sokoloski in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This study was supported in part by a grant from Parke, Davis and Co., Detroit, Mich.

Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.

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[1] C. H. Boehringer Sohn, West Germany, m.p. 215°, specific rotation −53°.

hydrogen ion concentrations were measured at room temperature on the Beckman model H pH meter using a glass indicator electrode with a reference calomel electrode.

A reaction vessel containing the solutions of epinephrine was so designed as to permit equilibration with oxygen at a particular partial pressure, withdrawal of samples without disturbing that atmosphere, stirring of the solution, and storage in a constant temperature bath at 65.0 ± 0.2°. Figure 1 shows this apparatus. After the introduction of the solution into the vessel the atmosphere above the liquid was evacuated and the entire apparatus allowed to come to temperature equilibrium. The reaction was considered initiated with exposure to the oxygen atmosphere.

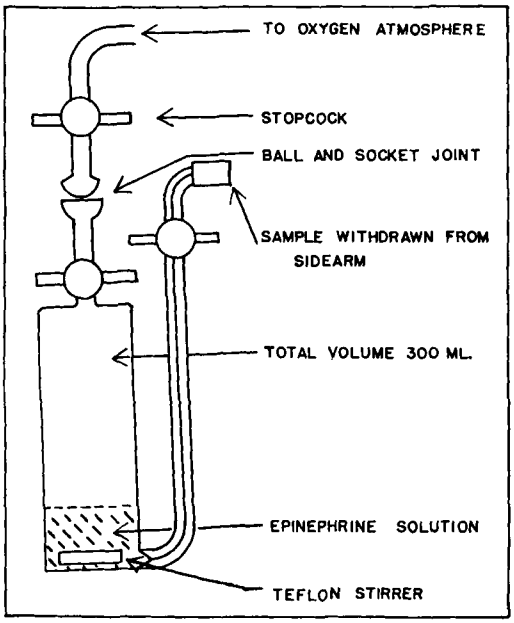


Fig. 1.—Schematic representation of the reaction vessel used in studying the rate of degradation of epinephrine at various drug concentrations and oxygen pressures.

A 5-gallon jar served as the oxygen source and was attached to the apparatus by glass tubing. By displacing known volumes of water with high purity oxygen and nitrogen, any desired partial pressure of oxygen could be obtained. A mercury manometer was attached to the system and the oxygen-nitrogen mixtures were maintained at a positive pressure of 3 to 4 cm. of mercury. This manometer served as a check to insure the maintenance of a constant oxygen pressure throughout any particular determination. The amount of oxygen added was such that if all of the epinephrine present were to degrade, there would be no more than a 15% decrease in the pressure above the solution. The error represented a maximum that was never realized since the epinephrine never completely degraded nor did the manometer indicate such a drastic change in pressure. For those studies above one atmosphere, the reaction vessel was attached directly to the oxygen tank through a trap and the gauge reading taken as the pressure above the solution.

After suitable lengths of time, samples of the desired volume were withdrawn quantitatively from the sidearm of the vessel into a pipet. The solution was then transferred to a beaker and frozen until the time of assay by the modified U.S.P. XV procedure (7). The amount of solution withdrawn depended on the concentration of the epinephrine and attempts were made so that the acylated solution initially contained the equivalent of 10 mg. of epinephrine prior to its transfer to a chromatographic column. For example, in the analysis of the 0.2 *M* epinephrine solution, 3 ml. of the solution was withdrawn, diluted to 25 ml. with distilled water, and 2 ml. of this solution acylated after adjusting the final volume to 5 ml. The acylated mixture was transferred to a column for chromatographic separation after mixing with 5.6 Gm. of Celite 545 as described in a previous study (7).

RESULTS

The dependence of the reaction with respect to the drug concentration was found to vary with the oxygen tension present and ranged from a simple proportionality at low oxygen pressures to a half-order at higher oxygen concentrations. When the oxygen tension was held at 0.13 atmospheres and the concentration of epinephrine followed as a function of time, plots represented by Fig. 2 were obtained. It should be noted that in this particular determination and in each of the other kinetic studies made, the degradation proceeded with no apparent lag phase. Reactions which follow a free radical path are often characterized by an initial lag time during which period intermediates accumulate.

Since it was not possible to determine clearly the dependency of the reaction with respect to the substrate by merely plotting various functions of the concentration against the time, the changes in the initial rate with initial concentrations of the drug

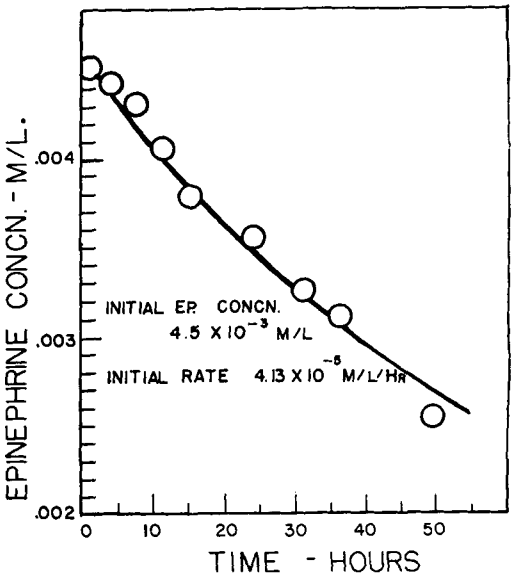


Fig. 2.—Epinephrine concentration (moles/L.) as a function of the time (hr.) at 0.13 atmosphere oxygen, pH 5, ionic strength 0.5 μ, and temperature 65° where the initial epinephrine concentration was 4.5 $\times 10^{-3}$ *M*.

TABLE I.—EFFECT OF EPINEPHRINE CONCENTRATION ON THE INITIAL RATE OF DRUG DEGRADATION AT VARIOUS OXYGEN PRESSURES

Oxygen Pressure, Atmospheres											
0.13		0.26		0.53		1.1		2.0		2.6	
A[a] × 10^3	B[a] × 10^5	A × 10^3	B × 10^4	A × 10^3	B × 10^4	A × 10^3	B × 10^4	A × 10^3	B × 10^4	A × 10^3	B × 10^3
4.5	4.15	7.00	1.70	4.8	2.7	4.10	9.5	4.44	7.5	4.90	1.51
5.03	6.20	50	10.7	9.6	3.76	9.70	11.8	4.78	10.4	9.40	2.40
10.3	11.4	21.3	4.60	46.4	13.2	50.5	19.8	9.93	18.7	49.0	5.90
11.0	10.8	170	29.3	183	33.4	51.8	21.0	16.6	28.8	192	7.30
43.6	67.5	...	...	...	...	45.8	21.3	47.5	43.3	...	...
...	...	...	...	...	...	354	75.0	186	95.0	...	...
...	...	...	...	...	...	...	...	188	67.5	...	...

[a] A = Initial epinephrine concentration (moles/L.); B = initial rate (moles/L./hr.)

TABLE II —FIRST-ORDER RATE CONSTANTS IN THE DEGRADATION OF EPINEPHRINE AT 0.13 ATM. OXYGEN

Initial Epinephrine Concn (moles/L × 10^3)	Graphically Obtained Rate Constant × 10^2 (hr.$^{-1}$)
4.51	1.1
5.03	0.9
10.3	1.0
11.0	1.0
43.6	1.5
183	1.1

were determined. The approach largely obviates participation of various degradation products in the epinephrine reaction *per se*, since these are assumed to be quite low in concentration in the beginning.

The initial rates were found by taking initial slopes of lines as shown in Fig. 2 expressed as moles/L./hr. By varying the initial concentration of the drug, the change in the initial rate as a function of drug concentration could be determined. The results of these determinations at 0.13 atmospheres oxygen appear in the first column of Table I. To determine the dependency of the rate with respect to the epinephrine concentration at this oxygen tension the data appearing in Table II were plotted on a log-log scale. It is evident in this type of plot that the slope of the line is identical with the dependency of the rate with respect to drug concentration.

By choosing six different oxygen pressures above the solutions and applying the same principles it was possible to determine the effect that oxygen had on the dependency with respect to epinephrine. These data are also summarized in the columns of Table I. The log-log plots establishing the dependency of epinephrine are given in Fig. 3. The manner in which the drug dependency is affected by the oxygen pressure is represented in Fig. 4. In this plot the order of the reaction with respect to the epinephrine concentration is shown as a function of the oxygen pressure and it is seen that the order changes quite rapidly initially as the oxygen pressure increases. At a value of about one atmosphere the order tended to level off and remained steady at about a half-order dependence.

At lower oxygen pressure the order with respect to the substrate is apparently first order. If the data of Fig. 2 were plotted on a semilogarithmic scale a straight line should be obtained, provided that degradation products did not alter the rate of reaction.

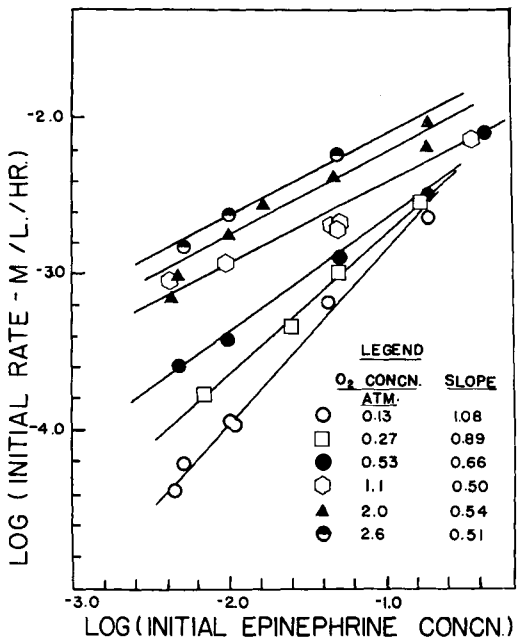


Fig. 3.—The logarithm of the initial rate of epinephrine degradation as a function of the logarithm of the initial epinephrine concentration at different oxygen pressures.

The logarithmic plot is presented in Fig. 5, and it is evident that the first-order dependency of the drug holds over a considerable loss of the substrate. The first-order rate constants calculated from plots as Fig. 5 should be independent of the initial epinephrine concentrations. Table II shows these rate constants obtained for different runs made at several initial concentrations of the drug. The variation observed here was only about 10% and well within the deviation to be expected.

The rate dependency of the catecholamine oxidation on oxygen tension above the solution was determined from data already presented in the preceding section. Since the initial rates of the loss of epinephrine having exactly the same initial epinephrine concentration were not determined under different oxygen tensions it was necessary to obtain these points by interpolation of such data as has been obtained. Each initial rate at a particular oxygen pressure and initial epinephrine concentration was interpolated from initial rates obtained for given different initial epinephrine concentrations

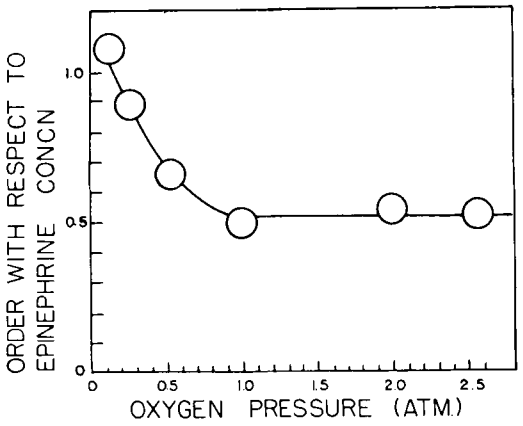


Fig. 4.—The order of the rate of epinephrine autoxidation with respect to drug concentration as a function of oxygen pressure (atmosphere).

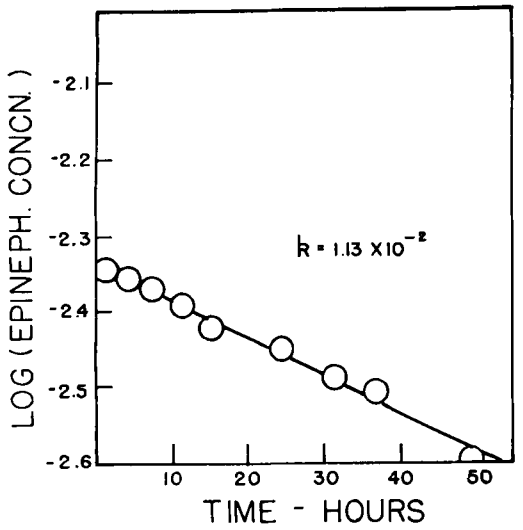


Fig. 5.—The logarithm of the epinephrine concentration as a function of the time (hr.) at 0.13 atmosphere oxygen, pH 5, ionic strength 0.5 μ, and a temperature of 65° where the initial epinephrine concentration was 4.5×10^{-3} M.

making use of Fig. 3. In this way the rates at six different oxygen pressures all at the same initial epinephrine concentration were found. Data were thus obtained for seven different initial epinephrine concentrations ranging from 0.0032 to 0.2 M. The interpolated data are shown plotted logarithmically in Fig. 6 as the initial rate against the oxygen tension. The slope of each line corresponds to the dependence of the autoxidative rate with respect to oxygen concentration at various initial epinephrine concentrations. It is apparent from Fig. 6 that as the concentration of epinephrine is increased, the slope or order with respect to oxygen concentration decreases. The apparent order is strongly affected by the epinephrine concentration as shown in Fig. 7, decreasing gradually at the higher levels of the drug. A fractional dependency with respect to oxygen concentration in an oxidative process is apparently not uncommon.

The effects of solutions of epinephrine initially at

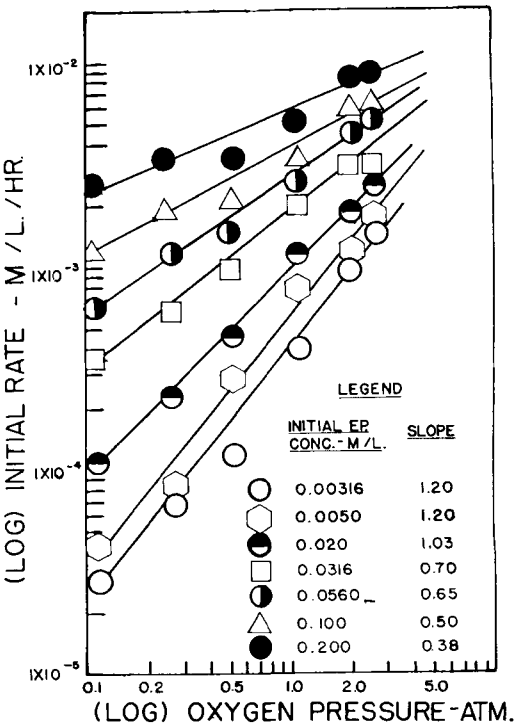


Fig. 6.—The logarithm of the initial rate of epinephrine degradation as a function of the logarithm of the oxygen pressure at different initial concentrations of epinephrine.

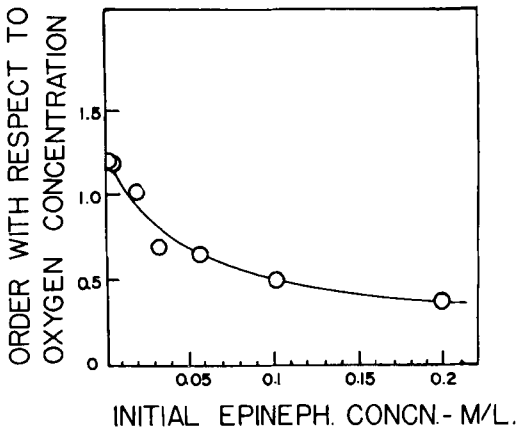


Fig. 7.—The order of the rate of autoxidation of epinephrine with respect to oxygen concentration as a function of the epinephrine concentration.

0.01 M and an ionic strength of 0.5 containing concentrations of 6.8×10^{-3} M potassium chloride, 2.2×10^{-4} M copper (II) acetate, and no copper or cyanide were compared. Oxidation appeared to take place even in the absence of copper although there can be no question that copper acted as an effective catalyst for epinephrine in aqueous solutions under the influence of oxygen. The graphs showing the dependence of the epinephrine concentration as a function of time did not exhibit a lag phase and thus were similar to those obtained in the epinephrine dependence study. The data

TABLE III.—EFFECT OF POTASSIUM CYANIDE AND COPPER (II) ACETATE ON THE DEGRADATION OF EPINEPHRINE

	Concentration of Additive, moles/L.	Half-Order Rate Constant $\times 10^2$, $(\text{moles/L.})^{1/2}$hr.
KCN	6.8×10^{-4}	1.0
KCN	6.8×10^{-3}	1.0
KCN	3.4×10^{-2}	1.2
KCN	6.8×10^{-2}	1.1
$Cu(OAc)_2$	2.2×10^{-4}	3.6
	0.00	0.8

yielded linear half-order plots. The resulting rate constants for each of the three solutions, potassium cyanide, copper acetate, and no copper or cyanide, are shown in Table III. It can be seen that the copper-containing solution degraded almost four times faster than the one containing potassium cyanide, and five times that of the one containing no copper or cyanide.

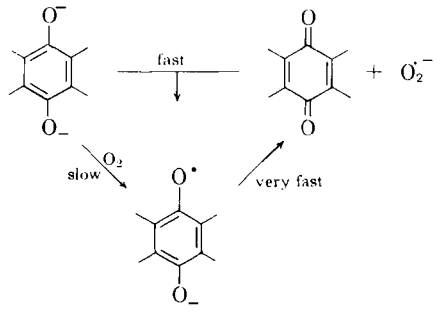
The rate of degradation of the solution containing the cyanide salt was more rapid than that containing none. This fact prompted a study of the effect of varying potassium cyanide concentrations investigating some possible catalytic action. The data, also shown in Table III, indicates, however, that even though the concentration of potassium cyanide was increased a hundredfold, no increase in rate constant was noticed.

DISCUSSION

The results of the studies on the kinetics of autoxidation of epinephrine as presented in the previous section are essentially summarized in Table IV. Orders referred to in the table were obtained from the dependencies of the initial rates of disappearance of the amine from solution.

At low concentrations of both reactants, the degradative reaction seems to be effectively second order. This simple dependence of the rate of reaction is not surprising but does not necessarily imply that the underlying mechanism is simple. Although Joslyn and Branch (8) reported that the oxidation of catechol proceeded with a first-order dependency on catechol concentration, this direct proportionality was apparently the consequence of a highly complex mechanism. Close analogies of the catechol degradation to the epinephrine oxidation cannot be made since in the case of epinephrine the intermediate species apparently involve the nitrogen. According to Wang (3) the rate of oxygen uptake is markedly reduced if a methyl radical replaces the primary nitrogen on epinephrine.

If the concentration of epinephrine and oxygen are both high, the rate of drug oxidation depends on the oxygen concentration raised to a fractional power and varies directly with the square root of the epinephrine concentration. The appearance of fractional orders strongly suggests that the degradative mechanism of epinephrine oxidation may be free radical mediated. This behavior is similar in some ways to the autoxidation of durohydroquinone, 2,3,5,6-tetramethyl hydroquinone, studied by James and Weissberger (9). According to their postulations the following scheme is indicated for this system



Such a scheme would rationalize the one-half power dependency of epinephrine at higher concentrations but, in turn, requires some sort of lag phase for the buildup of the quinoid species and a catalytic activity on the part of the oxidation products of the catecholamine. The present study gives neither any evidence of a lag phase nor data which suggests that the reaction is autocatalytic. The reaction seems to be totally unaffected by the accumulation of products during its course. This observation does not, of course, preclude the possibility that some highly unstable but dimerizable intermediate may play a catalytic role.

It should be pointed out that another work based on oxygen uptake by Trautner and Bradley (4) obtained evidence for major catalytic effects on the part of adrenochrome. The present results do not suggest that intermediate species cannot act catalytically but that these do not accumulate significantly in these solutions. It is interesting to note the part that the hydrogen ion concentration may contribute to the accumulation of intermediates. From experimental evidence it has been shown that an equilibrium exists between hydroquinone, quinone, and semiquinone according to the following scheme

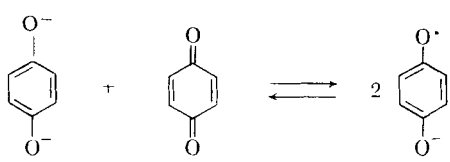


TABLE IV.—DEPENDENCE ON OXYGEN AND EPINEPHRINE CONCENTRATION OF THE INITIAL RATE OF EPINEPHRINE DISAPPEARANCE FROM SOLUTION AT HIGH AND LOW EPINEPHRINE AND OXYGEN CONCENTRATIONS

	Case	Order with Respect to Reactant: Oxygen	Order with Respect to Reactant: Epinephrine
I	Low epinephrine and low oxygen concentration	1^+	1.0
II	High epinephrine and high oxygen concentrations	Less than 0.5	0.5
III	High epinephrine and low oxygen concentration	Less than 0.5	1.0
IV	Low epinephrine and high oxygen concentration	1^+	0.5

The equilibrium favors a semiquinone formation when the divalent hydroquinone is the reactive species present, but in an acid solution the semiquinone is unstable and the reaction shifts considerably to the left. On the basis of these results, the studies of Trautner at pH 6–8 would apparently favor a stable semiquinone intermediate in contrast to the present studies made at pH 5 where the radical may possibly be more reactive and in lower concentration.

The results of Wang would tend to support the postulated rapid formation of radicals and may explain the apparent absence of a lag time. Her studies (3) show that oxygen was consumed in relatively short periods of time in comparison to the loss of epinephrine in the present investigation. At pH 4.9, 50°, and an epinephrine concentration of 0.001 *M*, Wang reports an oxygen consumption of 22.8 μl. after 0.6 hours, where a total of 224 was consumed after 9 hours.

It is possible to compare the results of the present investigation based on a chemical assay for the intact drug with that of Wang. By relating the rate of oxygen consumption to the rate of disappearance of epinephrine, Wang showed no lag phase present at pH 4.9 in a temperature range of 38 to 50°. However, at higher pH values a lag time was experienced. By interpolation of Wang's data the rate at which oxygen was consumed at 65° under air, where the initial epinephrine solution was 0.001 *M* at pH 4.9, was 9.5×10^{-6} moles/L./hr. By interpolating the data of the present study at approximately the same conditions, 0.001 *M* of epinephrine initially at pH 5 under 0.27 atm. oxygen, the rate was found to be 3.2×10^{-5} moles/L./hr. A close but not exact agreement suggests that perhaps the rate at which oxygen is consumed is some multiple of the rate of disappearance of the drug.

Under those conditions of high epinephrine concentration and low oxygen tension, the rate varies with a fractional-order dependency of oxygen and a first-order dependence with respect to epinephrine concentration. In contrast to this behavior we have the fourth case in which oxygen dependency is greater than unity and epinephrine is of a fractional order at low epinephrine and high oxygen pressure. Undoubtedly both oxygen and epinephrine are important reactants in the oxidation although their exact roles are not known. It is especially difficult to postulate the role of oxygen since indications are that, at low epinephrine concentrations, the oxygen dependence is greater than unity. There have appeared in the literature reactions which depend on a three-halves order with respect to oxygen but these were generally reactions in which metals were present as catalysts. In the present study oxygen dependence is shown only as a function of epinephrine concentration.

Chaix, Chauvet, and Jezequel (5) have said that in the absence of heavy metals an oxidation of epinephrine will not take place. The present studies have shown that although copper catalyzes the autoxidative degradation, the reaction probably takes place in their apparent absence. When the effect of various potassium cyanide concentrations was investigated, a small increase in rate over those solutions with no cyanide or copper was noticed, rather than the expected decrease. The cyanide has been known to act as a strong complexing agent for heavy metals and it was expected that it would act as a scavenger for the metals present in our system. The metals thus removed should have been unavailable for catalytic action. The small increase in rate is perplexing but demonstrates that heavy metals may not be needed for autoxidation.

Another evidence which suggests that trace amounts of metals in systems of the present study were not responsible for the degradation of epinephrine is based on the reproducibility that could be obtained. From Chaix's data an increase of copper ion concentration from 0 to 10^{-6} *M* or from 10^{-6} to 10^{-5} *M* would increase the rate of oxidation by a factor of two. Such increases in systems where the heavy metal concentration was not closely controlled would markedly affect reproducibility. The origin of free radicals may possibly come from oxygen and the organic species present and need not be initiated by heavy metals.

CONCLUSION

We are not prepared on the basis of the present work to present a complete mechanism sequence by which the observed results can be explained. The results suggest, however, that the overall reaction is an extremely complex one and, without question, involves free radical mediated sequences. The study appears to put to rest a number of previously unsupported concepts concerning the mode of oxidation of epinephrine at hydrogen ion concentrations approaching those commonly employed.

The behavior of this system as reflected in this study strongly points to formation of nonpersistent, dimerizable odd electron catalytic species. These probably act as the initial oxygen transfer agents. The results suggest, however, that none of the gross oxidation products possesses any significant catalytic effect.

From a purely practical and pharmaceutical standpoint it is evident from the data presented that for systems exposed to high oxygen tension the rate of degradation is always less than first order and that the percentage of the drug lost per unit time is always less per unit time at higher concentrations than at lower concentrations. These and other conclusions possible from these results may be of value in formulation of the drug.

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Determination of *m*-Aminophenol (MAP) in *p*-Aminosalicylic Acid (PAS) and Sodium *p*-Aminosalicylate (PASNa)

By R. B. LUERS, Jr., and L. B. STADLER

The MAP test in the U.S.P. XVI monographs for PAS and PASNa has been re-evaluated. Experimental data based upon paper chromatography showed the test to be sound but in need of minor changes to correct sources of error. As a result of this study, recommendations are made to change the correction factor, to determine maximum absorbance through a range of 420 to 435 mμ, and to change the time allowed for development of color to 3 hours.

THE MAP TEST in the U.S.P. XVI monographs for PAS and PASNa (1) is based upon a color development shown by Pesez (2) to be the result of a series of diazotization coupling reactions. Seaman, *et al.* (3), refined the method which Pesez had published. The color reactions are comprehensively reviewed by Feiser and Feiser (4). The U.S.P. XVI test for MAP is basically a sound analytical procedure; however, experience has indicated that certain discrepancies exist in the test. It has been recognized for some time that absorbance values in the MAP test often were less than 0.372 (the quantity alleged to represent color formed by PAS itself in the test), resulting in apparent minus percentage values for MAP content. Furthermore, the absorbance values decrease during the first 3 hours after color development possibly as the result of a decrease in background color. In an effort to understand the apparent variables in this test for MAP better, an investigation was undertaken to study the various conditions involved in the U.S.P. XVI test for MAP.

In order to establish the absolute MAP content of some production samples of PAS and PASNa, a paper chromatographic procedure was developed in our laboratory. Briefly, the procedure consisted of chromatographic separation of the MAP from the PAS and PASNa using a benzene and methanol mobile solvent and the quantitative estimation of MAP by a spectrophotometric ultraviolet scanning technique.

EXPERIMENTAL

Proposed Modified Colorimetric Method for MAP in PAS and PASNa.—The proposed revisions of the U.S.P. XVI test for MAP, as described under *Procedures*, were performed on samples of PAS and PASNa. The basis for the revisions proposed are discussed under *Conditions Affecting the Colorimetric Determination of MAP*.

Reference PASNa.—The reference PASNa used in this investigation was a sample that was shown to contain less than 0.01% of MAP, as determined by the paper chromatographic method. When tested by the proposed revised colorimetric method described under *Procedures*, the reference PASNa showed an absorbance of 0.320. Repeating this test on the reference PASNa through a period of 20 different days, a range of absorbance values of 0.319 to 0.322 was obtained. In view of this, it was concluded that this sample represented reference quality PASNa in conjunction with this study.

A specially prepared sample of PASNa received from Dr. L. H. Welsh,[1] when tested by the paper chromatographic method and by the proposed revised colorimetric method, compared very closely with the reference PASNa used in this study.

CONDITIONS AFFECTING THE COLORIMETRIC DETERMINATION OF MAP

Effect of Time upon the Color Intensity Developed.—As discussed by Seaman, *et al.* (3), the time after addition of the sulfuric acid and sodium nitrite is quite critical. As the result of our investigations, it was found that after the addition of the sodium carbonate solution the intensity of the color decreased rapidly over a 3-hour period. After 3 hours the color intensity stabilized (See Fig. 1.). For this reason the time lapse between the addition of the sodium carbonate solution and the measurement of color intensity has been changed to 3 hours in the proposed modified colorimetric method described under *Procedures* for MAP determinations in PAS and PASNa. This characteristic of the color development was found evident in all samples of PAS and PASNa tested.

Effect of Change in Concentration of MAP upon the Absorbance Maximum of the Color Developed.—To confirm the absorbance peak of 440 mμ for the MAP color intensity produced by the U.S.P. XVI method, absorption spectra in the 350 to 700 mμ range were obtained from two samples of PASNa known to contain 0.20% and less than 0.01% of MAP, using a Cary recording spectrophotometer

Received February 24, 1961, from the Analytical Laboratories, Parke, Davis and Co., Detroit 32, Mich.
Accepted for publication May 2, 1961.
The authors are greatly indebted to Mr. F. A. Maurina for guidance and technical assistance given. The authors wish to express their gratitude to Mr. Carl E. Greco and Mr. Robert Eppler for paper chromatography, Miss Carolyn Holtrey and Mr. Richard Zingle for spectrophotometric curves, and to Dr. J. M. Vandenbelt and staff for spectrophotometric scanning of chromatographic strips.

[1] Department of Health, Education and Welfare, Food, and Drug Administration.

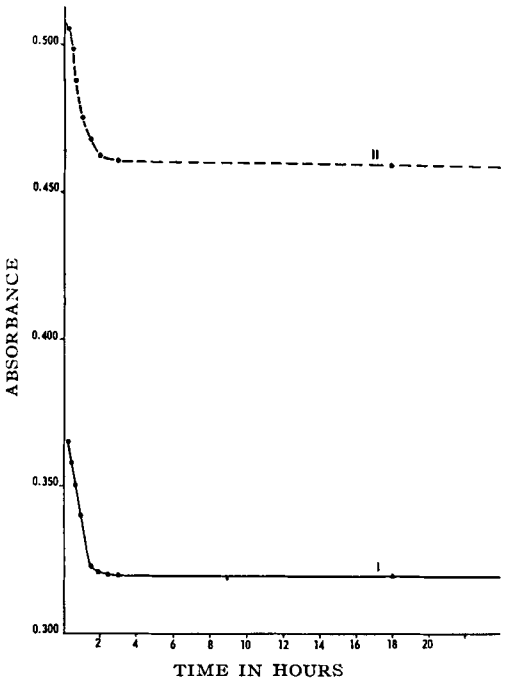


Fig. 1—Relationship of absorbance and time for PASNa samples. I, Reference quality PASNa containing less than 0.01% MAP; II, PASNa containing 0.12% MAP.

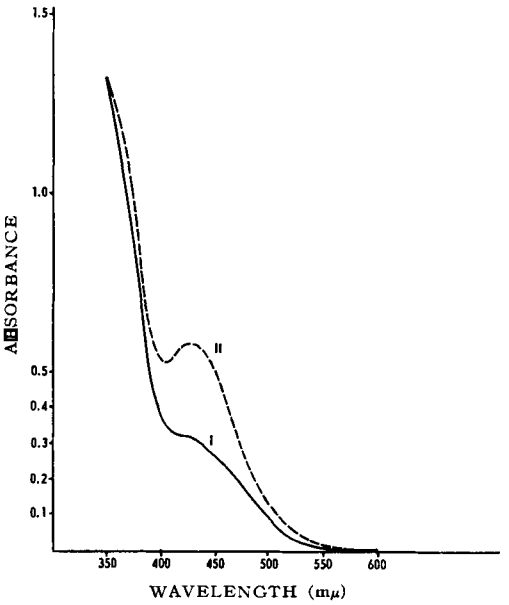


Fig. 2—Relationship of MAP concentration and absorption maxima. I, Reference quality PASNa containing less than 0.01% MAP; II, PASNa containing 0.20% MAP.

TABLE I.—EFFECT OF CHANGE IN CONCENTRATION OF MAP UPON ABSORBANCE MAXIMUM

Per cent of MAP in PASNa	Absorbance Maximum Found, mμ	Comment on Curve Characteristics
0.02	420–425	Plateau occurs
0.04	420–425	Plateau occurs
0.08	425	Round curve occurs
0.10	425	Sharper peak occurs
0.15	430	Sharp peak occurs
0.20	430	Sharp peak occurs
0.30	430	Sharp peak occurs
0.50	435	Sharp peak occurs
1.00	435	Sharp peak occurs

(model 11). As indicated by Figure 2, the PASNa containing less than 0.01% of MAP showed a plateau-like peak in the 420–425 mμ range, and the PASNa containing 0.20% of MAP showed a peak at 430 mμ. As a result, a series of determinations were conducted, using solutions of PASNa containing known quantities of MAP ranging from 0.02 to 1.0%. The absorbance maxima obtained for these concentrations of MAP are shown in Table I. These results indicate a shift in the absorbance maximum with increasing MAP concentrations and justify the use of a 420 to 435 mμ range for the measurement of the maximum absorbance of the color developed by the proposed modified colorimetric method described under *Procedures* for MAP in PAS and PASNa.

Based upon our study of a number of production samples of PAS and PASNa and the correlation of data between the proposed modified colorimetric method and paper chromatographic procedure (see Table II), the correction value for color produced other than by MAP should be 0.320 rather than 0.372. The absorbance values of our reference PASNa and the PASNa sample from Dr. Welsh also support the 0.320 value.

PROCEDURES

For PAS.—Weigh accurately 500 mg. of PAS and transfer to a 100-ml. volumetric flask with the aid of 5 ml. of sodium hydroxide T.S. and 5 ml. of water. Mix to dissolve the sample and dilute the solution with water to about 80 ml. Add 10 ml. of dilute sulfuric acid (1 in 10), add water to volume, and mix. Within 150 seconds from the time the acid is added, transfer 5 ml. of this solution to a second 100-ml. volumetric flask immersed in an ice bath and containing 50 ml. of water at 0 to 5°, and add 2.5 ml. of sodium nitrite solution (1 in 100). Mix and allow to stand in the ice bath for 3 minutes ± 5 seconds. Add 25 ml. of sodium carbonate T.S., mix, and place the flask in a water bath at 25° for 15 minutes. Add water to volume, mix, and allow the solution to stand at 25° for 3 hours. Determine the absorbance of the solution at the absorbance maximum observed in a range of 420 to 435 mμ in a 1-cm. cell with a suitable spectrophotometer, using water as the blank. Calculate the per cent of MAP by the formula $(A - 0.320)/0.84$, in which A is the absorbance of the solution, 0.320 is the absorbance correction factor representing color produced other than by the reaction of MAP initially present, and 0.84 is the factor converting absorbance to per cent of MAP in PAS.

For PASNa.—Weigh accurately 690 mg. of PASNa and transfer to a 100-ml. volumetric flask. Add 80 ml. of water and 1.8 ml. of sodium hydroxide

TABLE II.—PER CENT OF MAP FOUND BY THE DIFFERENT METHODS TESTED

Sample of PASNa Tested	Per cent MAP Found: By Paper Chromatography	By U.S.P. XVI Method	By Proposed Modified Method
Reference Sample	Less than 0.01	Negligible[a]	Less than 0.01
Sample A	0.04	Negligible	0.03
B	0.12	0.08	0.12
C	0.01	Negligible	0.01
D	...	Negligible	0.07
E	...	Negligible	0.04
F	...	Negligible	0.04
G	...	Negligible	0.03
H	...	Negligible	0.04
FDA Sample	Less than 0.01	Negligible	Less than 0.01
Sample of PAS Tested			
Sample A	0.06	Negligible	0.05
B	1.25	1.17	1.21
C	0.65	0.57	0.65
D	0.02	Negligible	0.03
E	...	0.03	0.10
F	...	Negligible	0.07
G	...	Negligible	0.05
H	...	Negligible	0.06
J	...	Negligible	0.04
K	...	Negligible	0.08
L	...	Negligible	0.09
M	...	0.05	0.12
N	...	Negligible	0.03
P	...	0.04	0.11
Q	...	0.01	0.09

[a] The term negligible denotes that the absorbance value for the sample was less than 0.372, which by the U.S.P. XVI method would result in a minus percentage value for MAP content.

T.S., and mix. Add 10 ml. of dilute sulfuric acid (1 in 10), add water to volume, and mix. Within 150 seconds from the time the acid is added, transfer 5 ml. of this solution to a second 100-ml. volumetric flask immersed in an ice bath and containing 50 ml. of water at 0 to 5°, and add 2.5 ml. of sodium nitrite solution (1 in 100). Mix and allow to stand in the ice bath for 3 minutes ± 5 seconds. Add 25 ml. of sodium carbonate T.S., mix, and place the flask in a water bath at 25° for 15 minutes. Add water to volume, mix, and allow the solution to stand at 25° for 3 hours. Determine the absorbance of the solution at the absorbance maximum observed in a range of 420 to 435 mμ in a 1-cm. cell with a suitable spectrophotometer, using water as the blank. Calculate the per cent of MAP by the formula (A − 0.320)/1.16, in which A is the absorbance of the solution, 0.320 is the absorbance correction factor representing color produced other than by the reaction of MAP initially present, and 1.16 is the factor converting absorbance to per cent of MAP in PASNa.

SUMMARY AND CONCLUSIONS

1. The MAP content found by the proposed modified colorimetric method was comparable to that found by paper chromatography for the PAS and PASNa tested in this study.

2. The color produced by both the U.S.P. XVI method and the proposed modified colorimetric method stabilized after standing 3 hours at 25°.

3. The absorbance spectra obtained from the U.S.P. XVI method and the proposed method showed the absorbance maxima to be between 420 and 435 mμ.

4. The correction factor for color produced other than that by MAP should be changed from 0.372 to 0.320.

5. The values of 0.84 and 1.16 for conversion of absorbance to per cent MAP are correct.

6. The U.S.P. XVI procedure for the determination of MAP in PAS and PASNa is basically reliable.

7. It is recommended that the modifications, as herein described, be adopted by the U.S.P. XVI to correct minor sources of error in the determination of MAP in PAS and PASNa.

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In Vitro Testing of Timed Release Tablets and Capsules

By E. O. KRUEGER and E. B. VLIET

An apparatus, previously described (1), is recommended for *in vitro* testing of timed release dosage forms.

THE INCREASING use of timed release dosage forms (in particular, prolonged action tablets and capsules) was recognized by the Contact Section in the Fall of 1957. A special committee on timed release tablets and capsules was organized in the Spring of 1958 with the following objectives: to develop an apparatus and an *in vitro* procedure that might be generally useful as a routine control method to check the uniformity of drug release from lot to lot.

From the beginning, the committee limited its considerations to *in vitro* control procedures. Specifically, it has not attempted to develop methods that can be used in formulating timed release products. This must be done with appropriate *in vivo* studies.

During the past 4 years the committee has worked towards its objective through a series of collaborative efforts.

Much of the early stimulation and interest was provided by an apparatus and procedure used by Dr. Frank Wiley and his associates at the Food and Drug Administration (2). A description of this equipment and method was sent to interested PMA member firms with the request that they try it on their products. Laboratories that tried the method felt it was somewhat more complex and less convenient than is desired in a routine control method.

The subcommittee agreed that an effort should be made to determine whether the U. S. P. basket-rack assembly (U. S. P. XV, p. 937) might be adaptable as a suitable test apparatus. The first collaborative study using the modified U. S. P. equipment followed the Spring 1958 meeting of the Contact Section. Nine laboratories were equipped with metal cartridges designed to fit inside the tubes of the U. S. P. tablet disintegration apparatus. The cartridges were closed at each end with 30-mesh screens. Two products were tested. Data obtained from this study showed reasonable agreement among the collaborating laboratories.

As a result of comments arising from this work, slight modifications were made in the equipment and a second study was undertaken after the Fall 1958 meeting. In this study, metal cartridges of about the same size as the tubes in the U. S. P. tablet disintegration procedure were used to provide a better flow of the disintegrating fluid, and the 30-mesh screens were replaced with 40-mesh screens so as to retain finer particles better. The cartridges were supported on a basket-rack holder. Three different products were studied. Each of the products contained a single drug component readily soluble in water. The release pattern was determined in two ways: (*a*) by assaying the drug in the disintegrating fluid, and (*b*) by assaying the undisintegrated residue. This study showed good intra- and interlaboratory agreement in results, and established the interchangeability of determining the drug in the test fluid or in the residue. However, it was generally agreed that it was simpler to determine the drug in the residue, since this avoided any interference effects of the test fluid on the analytical procedures. A summary of this second study was published in *Drug Standards* (3).

At that time it was believed that the apparatus and method described in the second study might be generally useful and convenient for laboratory control of lot-to-lot uniformity for most timed release products.

One of the collaborating laboratories then studied this procedure further and compared the results obtained by using it with those by the rotating bottle method previously described in *Drug Standards* (1) and also with those found by using the initial procedure of the Food and Drug Administration laboratories (2). It became obvious that there were marked differences in release rates using the three methods, particularly with products containing sparingly soluble drugs.

Received November 11, 1961, from Abbott Laboratories, North Chicago, Ill.
Accepted for publication December 18, 1961.
Report of a special committee of the Contact Section of the Pharmaceutical Manufacturers Association, which included C. L. Graham, L. M. Lueck, C. M. Mitchell, A. Nutzul, E. B. Vliet, F. H. Wiley, and E. O. Krueger, chairman.

The rotating bottle method yielded values more usable for demonstrating timed release and completeness of release (hence, more suitable for control purposes) than did the other two methods.

As a result, a third study was set up testing four different products in eight laboratories. One product contained a readily soluble drug, whereas the other three contained drugs that were only sparingly soluble. Each sample was checked in each laboratory by five procedures as follows: (*a*) Rotating bottle procedure with a complete change from simulated gastric fluid T. S. to simulated intestinal fluid T. S. after 1 hour. (*b*) Rotating bottle procedure with a gradual pH change, accomplished by first using simulated gastric fluid T. S. and, at each subsequent time interval, withdrawing one-half of the fluid and replacing it with simulated intestinal fluid T. S. (*c*) Cartridge basket-rack procedure with a complete change from simulated gastric fluid T. S. to simulated intestinal fluid T. S. after 1 hour. (*d*) Cartridge basket-rack procedure with a gradual pH change effected as in (*b*). (*e*) FDA procedure.

The analyses were performed on the residue in methods *a*, *b*, *c*, and *d*, and on the extracting fluid in method *e*. The results from all laboratories on all four samples were in good agreement.

The product containing the freely soluble drug showed by all 5 procedures: (*a*) comparable results at all time intervals, (*b*) almost complete release after 8 hours, and (*c*) no differences in release, irrespective of whether the pH change was abrupt or gradual.

With the other three products the rate of release was greatest (nearly complete after 8 hours) with the rotating bottles; considerably less (from 35 to 65% released after 8 hours) with the cartridges and basket-rack; and least (from 10 to 50% released after 8 hours) by the FDA method. These differences seem to be in line with variation in the rate of flow of the fluids around the tablets or capsules. The flow is greatest in the rotating bottles, considerably less in the cartridges, and is quite restricted in the FDA apparatus. Results using the gradual pH change were, in general, only slightly different than those using the abrupt pH change technique.

It was decided to explore further the use of this *in vitro* rotating bottle procedure on a larger variety of timed release products to determine how extensively it might be applied and to ascertain whether the results obtained in different laboratories would be sufficiently consistent so that the method might be used for routine control testing.

THE NEW COLLABORATIVE STUDY

Scope.—In this study 12 different products were tested; 11 tablets and one capsule. Six had one drug component, while four contained two drugs each, one had three and one had four. There were at least five different types of release mechanisms in this group of samples and the drugs in the products varied from very soluble to sparingly soluble.

Eight laboratories participated in the study. All samples were sent to the laboratories on a coded basis along with the assay techniques to be used. These had been furnished by the firms that submitted the samples and were checked in other laboratories before and during the study. This, and the assays during the tests, entailed a great amount of work, especially in the multicomponent products. The release rates of each sample were generally determined in four different laboratories. One laboratory checked all of the samples.

Apparatus.—The apparatus used was described by Souder and Ellenbogen (1), was referred to in the previous report of this committee (4), and is shown in Figs. 1 and 2. Disintegration of samples is accomplished in 90-ml. bottles containing 60 ml. of disintegrating fluid revolving end over end in a 37° bath at 40–44 r. p. m.

Procedure.—It was realized that, in adapting a routine control testing method to a specific timed release product, the time intervals to be checked and the nature of the extracting fluid to be used would depend upon the release pattern the product was designed to give and upon the nature of the drugs and other components it contains. Nevertheless, it was decided that in this series of tests all laboratories would check the amount of drug released by all samples after intervals of 1, 2, 3.5, 5, and 7 hours. In all cases, simulated gastric fluid T. S. would first be used. At subsequent time intervals this would be replaced by mixtures of simulated gastric fluid T. S. and simulated intestinal fluid T. S. so that after 1 hour the pH would be 2.5, then 4.5 after 2 hours, and 7.0 after 3.5 hours. Simulated intestinal fluid T. S. at pH 7.5 would be used after 5 hours. The analysis would be performed on the residue in all cases.

The laboratory which tested all of the samples also checked each one using an abrupt pH change. Simulated gastric fluid was completely replaced by simulated intestinal fluid after the first hour.

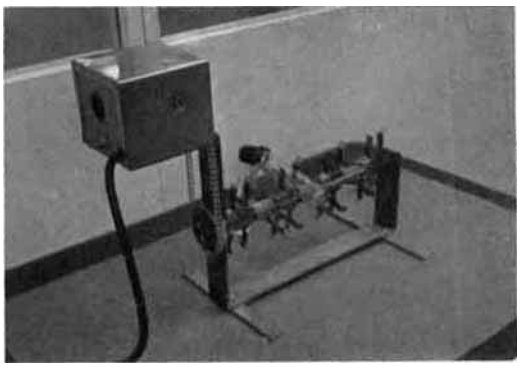


Fig. 1.—Rotating bottle apparatus. (Available from E. D. Menold Sheet Metal Co., 8214 Lisser Ave., Philadelphia, Pa. Bottles are not included.)

TABLE I.—*In Vitro* RELEASE OF DRUG FROM PRODUCT A, REPORTED BY FIVE LABORATORIES

Laboratory No.	Per Cent of Drug Released After Elapsed Times, hr.				
	1	2	3.5	5	7
2	44	50	60	68	78
5	45	54	66	70	82
1	51	58	69	76	84
6	45	50	56	66	68
4	47	60	67	73	82

TABLE II.—*In Vitro* RELEASE OF DRUG FROM PRODUCT B, REPORTED BY FOUR LABORATORIES

Laboratory No.	Per Cent of Drug Released After Elapsed Times, hr.				
	1	2	3.5	5	7
3	30	50	59	63	75
2	37	53	60	68	79
1	35	54	63	72	81
7	46	56	64	74	83

RESULTS

Results obtained in the different laboratories on each sample using the prescribed procedure were in reasonable agreement. When the procedure was modified by abruptly changing the pH of the fluid after 1 hour, the release rates of pH-sensitive drugs were altered, as would be expected.

Of the 12 products tested, only one failed to release its drug as expected. The method, as applied in this study, would seem to be unsuitable for such a product, which depends upon a release mechanism considerably different than those used in the other products studied.

Data shown in Tables I, II, III, and IV, are typical of those obtained in the study and indicate how well results from different laboratories agreed. Results on the other products studied showed the same general degree of agreement.

CONCLUSION

After carefully considering the results obtained in this and the preceding collaborative study, and after communicating with those supplying the samples tested, the special committee made an observation and a recommendation to the PMA Contact Section at its meeting in October 1961. These were unanimously accepted and approved by the membership of the Section and are as follows:

1. The apparatus described in *Drug Standards* (1) and shown in Figs. 1 and 2 is more useful for the *in vitro* control testing of most timed release products now marketed than any other apparatus studied to date.

2. In the event the U. S. P. and N. F. should

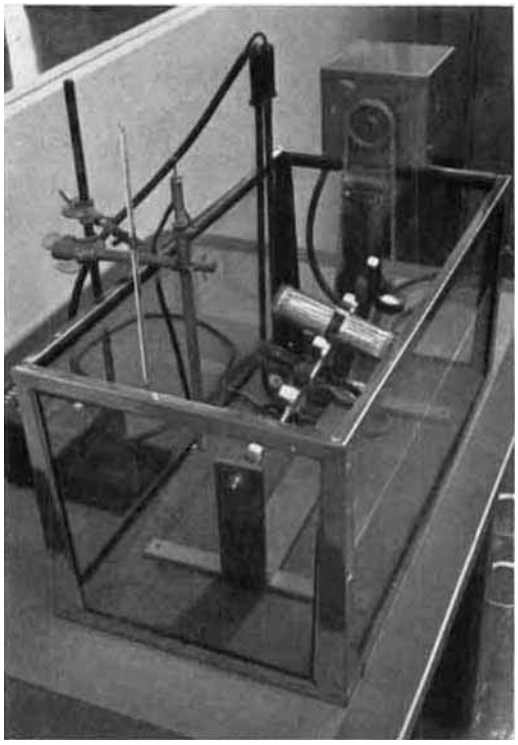


Fig. 2.—Rotating bottle apparatus and accessory equipment: immersion heater, mercury thermoregulator (37°), thermometer, standard relay, constant temperature bath, and bottles ($3^1/_8$ oz. round tablet, Owens-Illinois mold 5676).

TABLE III.—*In Vitro* RELEASE OF TWO DRUGS FROM PRODUCT C, REPORTED BY FOUR LABORATORIES

	Per Cent of Drug Released After Elapsed Times, hr.									
	1		2		3.5		5		7	
Drug component	S	T	S	T	S	T	S	T	S	T
Laboratory No. 1	57	31	79	66	87	84	93	95	99	97
2	61	27	77	46	88	73	91	88	97	97
4	63	29	79	48	88	69	91	85	96	96
3	54	30	73	44	85	68	87	86	94	97

TABLE IV.—*In Vitro* RELEASE OF TWO DRUGS FROM PRODUCT D, REPORTED BY THREE LABORATORIES

	Per Cent of Drug Released After Elapsed Times, hr.									
	1		2		3.5		5		7	
Drug component	X	Y	X	Y	X	Y	X	Y	X	Y
Laboratory No. 1	40	62	53	68	64	77	80	87	90	95
2	34	59	50	67	64	77	76	85	90	95
3	37	56	47	62	67	70	77	81	91	91

include a section on timed release products, it is recommended that this rotating bottle apparatus be considered for use for *in vitro* control testing of such products, with the understanding that the speed of rotation used on certain products may have to be changed to increase the sensitivity of the test for control purposes; and that the time intervals and the pH and other characteristics of the immersion fluids used, must be given individual consideration so as to be appropriate for each product tested.

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Notes

Effect of Ionized Air on Early Growth of Black Mustard Seedlings

By ROBERTSON PRATT

Mammalian systems, bacteria, and fungi have previously been reported to be affected by ionized air. The present paper records an effect of such air on a spermatophyte, black mustard *Brassica nigra* (L.) Koch. Neither positive nor negative air ions influence germination of the seeds, but both types of ions depress the early growth of roots and shoots of the seedlings.

NUMEROUS EFFECTS of ionized air on biologic systems, including human beings, have been reported for more than 200 years, and attempts to apply some of these effects clinically were made as early as 1754. However, most of the reports must be classed as testimonial or, at best, speculative. Within the past decade possibly more reliable, but nonetheless subjective, clinical appraisals have indicated an ameliorating effect of negative air ions and an aggravating effect of positive ions on sinusitis, rhinitis, asthma, pollenosis, and related conditions. The literature up to 1935 has been reviewed extensively (1) and the more recent literature briefly (2).

Among quantitatively measurable effects of air ions on other biologic systems are reduction in succinoxidase content of the adrenal gland of the intact rat exposed to positively ionized air (3); killing of staphylococci by both positive and negative air ions (4); increase in ciliary movement in mammalian trachea, both *in vivo* and *in vitro*, when exposed to negative air ions and the reverse effect, or even abolition of movement, when exposed to positive ions (5–7); and reduction of germination of spores, growth of mycelium and elaboration of penicillin in cultures of *Penicillium notatum* exposed to an atmosphere enriched with air ions, negative ions having a greater effect than positive ones in reducing production of penicillin but positive ions exerting the greater depressing effect on spore germination and mycelial growth (2). The data below, taken from a larger continuing study, record a quantitatively measurable effect of air ons on early seedling growth of a spermatophyte, black mustard (*Brassica nigra*).

Received October 31, 1961, from the University of California, School of Pharmacy, San Francisco.
Accepted for publication December 11, 1961.

EXPERIMENTAL

Seeds, surface sterilized by immersion for 20 minutes in a dilute solution of calcium hypochlorite, were rinsed several times in sterile distilled water, then were soaked for 1 hour in same, and finally were placed aseptically on moist sterile filter paper strips about 2 cm. wide adhering to the inner surfaces of the germination chambers, consisting of 600-ml. Pyrex beakers. The strips (placed one in each beaker about 6 cm. from the top) were kept moist by a wick which consisted of a "tail" about 1.5 cm. wide that extended to the bottom of the chamber where it dipped into sterile distilled water. Seeds of black mustard are small enough to adhere easily to moist filter paper, even on a vertical surface. Soaking the seeds in water not only removed traces of hypochlorite, it also improved adhesiveness. Experiments were conducted in diffuse daylight; the temperature ranged from 20 to 23°.

A sterile ion generator head,[1] which has been described elsewhere (2), was supported about 1 cm. below the top of each beaker, and then the beaker was aseptically closed with sterile aluminum foil. The details and theory of the ion generators have been reviewed (8–10). At the rectifier voltage employed (860 v.), the atmosphere of the closed beakers was enriched with about 9.5×10^6 ions of appropriate charge/ml./sec. Radiation controls, similar to those run in earlier experiments on *Penicillium* (2), failed to reveal any evidence of direct radiation effects on the seeds or seedlings.

The data in Table I (averages computed from 5 replicate experiments totaling 500 seeds in each of the four environmental conditions) indicate that

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include a section on timed release products, it is recommended that this rotating bottle apparatus be considered for use for *in vitro* control testing of such products, with the understanding that the speed of rotation used on certain products may have to be changed to increase the sensitivity of the test for control purposes; and that the time intervals and the pH and other characteristics of the immersion fluids used, must be given individual consideration so as to be appropriate for each product tested.

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TABLE I.—PER CENT GERMINATION OF SEEDS AND ROOT AND SHOOT LENGTH OF SEEDLINGS OF BLACK MUSTARD EXPOSED TO IONIZED AIR

Ionization	Per Cent Germination	Average Root Length, mm. 48 hr.	72 hr.	96 hr.	120 hr.	Average Shoot Length, mm. 72 hr.	96 hr.	120 hr.	Root/Shoot Ratio 72 hr.	96 hr.	120 hr.
None (no unit)	89.7	9.4	27.1	63.0	93.0	6.0	16.9	19.7	6.5	6.0	5.1
None (unit not connected to power)	89.2	9.8	28.0	63.2	92.3	5.5	17.6	20.1	6.9	6.2	5.4
Negative	90.1	6.6	22.3	46.1	67.7	2.9	12.5	16.5	12.3	7.2	4.1
Positive	89.6	6.1	22.4	45.8	65.2	3.0	12.2	16.4	12.0	7.3	4.4

germination of black mustard seeds was not affected by an atmosphere enriched with ionized air (at the level employed), but that subsequent early growth of roots and shoots of the seedlings was depressed. The root/shoot ratios (last 3 columns of Table I) were obtained by dividing the total length of roots by the total length of shoots. The high values at 72 hours for plants exposed to ionized air (first of the 3 columns) reflect the delay in emergence of shoots in these seedlings. But, during the next 2 days, the increase in shoot length with respect to increase in total root length was greater for seedlings exposed to air ions than for control seedlings. Thus, the early differences in root/shoot ratio were eliminated, and there is indication of a reversal in the trend by the fifth day. For technical reasons, it was not practical to continue measurements beyond the fifth day.

DISCUSSION

Positive and negative air ions exerted qualitatively and quantitatively similar effects on seedlings of black mustard. This finding is similar to results obtained with staphylococci (4) but contrary to observations on succinoxidase content of rat adrenal gland (3), on ciliary movement in mammalian trachea (5–7), and on spore germination, mycelial growth, and penicillin production in cultures of *Penicillium* (2), in all of which significant differences were noted between the effects of negatively and positively charged air ions.

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Effect of Small Variations in Heat Stimulus Temperature on the Tail Flick Response of Rats in Analgesimetry

By J. JUSZKIEWICZ-DONSBACH and GERHARD LEVY

The heat-induced tail flick reaction time of rats is affected by small changes in temperature of the heat stimulus. A crossover experiment, using temperatures of 48 and 50°, respectively, showed significant differences in reaction times in a group of 31 rats. The results demonstrate the need for accurate temperature control of heat sources.

VARIOUS METHODS employing thermal stimuli are used widely in some phases of the evaluation of analgesics (1). Such techniques include the use of electric hot plates, hot wires, and metal tubing heated by circulating hot water. In our hands, the determination of the reaction time after immersion of the rat's tail in a beaker of hot water maintained at constant temperature proved to be the most reproducible method.

In many instances, the thermal sources used in the methods cited above maintain a given temperature within a range of ±1 or ±0.5°. During preliminary experiments we found that such relatively small fluctuations in temperature of the heat stimulus appear to have a significant effect on the reaction time of the animals. In order to investigate this factor in greater detail, the heat-induced reaction time of a number of rats was determined at 48 and at 50°. Both temperatures were maintained within ±0.05°. It was also of interest to determine the effect of repeated stimuli at 15-minute intervals on reaction time as well as the likelihood of "conditioning" due to regular application of the heat stimulus over a period of several weeks.

EXPERIMENTAL

Female Wistar rats weighing 50–90 Gm., starved for about 12 hours, were placed in individual plexiglas holders (Fisher Scientific Co., item 1-280) for 30 to 60 minutes. After an animal had calmed

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In many instances, the thermal sources used in the methods cited above maintain a given temperature within a range of ±1 or ±0.5°. During preliminary experiments we found that such relatively small fluctuations in temperature of the heat stimulus appear to have a significant effect on the reaction time of the animals. In order to investigate this factor in greater detail, the heat-induced reaction time of a number of rats was determined at 48 and at 50°. Both temperatures were maintained within ±0.05°. It was also of interest to determine the effect of repeated stimuli at 15-minute intervals on reaction time as well as the likelihood of "conditioning" due to regular application of the heat stimulus over a period of several weeks.

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down, one-third of the length of its tail was immersed in a beaker of water maintained at either 48 or 50 ± 0.05°. The beaker was an especially constructed, double-wall container of 400-ml. capacity, which permitted the circulation of water at constant temperature with a circulatory pump connected to a thermostatically controlled water bath. The water in the beaker was agitated with a magnetic stirrer to reduce any temperature gradient. The magnetic stirrer was stopped immediately prior to and during the time an animal's tail was immersed.

The animals reacted to the thermal stimulus with a typical contraction of their tails into circles. The time of this reaction was measured with a stop watch which was turned away from the operator to reduce a possible subjective influence upon him. Reaction times were determined at 15-minute intervals for a total of three determinations. The same individual carried out all measurements in order to assure proper interpretation of the end point. From 4 to 8 animals were used at a time. Half of these received a 50° heat stimulus; the others were subjected to a heat stimulus of 48°. Two days later the conditions were reversed, so that the response of each animal could be measured at both temperatures.

A number of animals were subjected to the described thermal stimulation twice a week for three weeks in order to determine a possible conditioning of their response.

RESULTS AND DISCUSSION

The first-week data obtained from several groups, totaling 31 animals, are shown in Table I. A significant difference ($P < 0.01$) in response to the 48° as compared to the 50° heat stimulus was observed. The reaction times during the third week of animals subjected to heat stimulation twice weekly for three weeks are listed in Table II. No significant differences between the first week and the third week reaction times of these animals at a given temperature were found, but the results obtained during the third week again show the effect of a 2° temperature difference on reaction time. It appears that the animals are not "conditioned" by repeated stimulation over a period of three weeks. This is in agreement with the findings of D'Amour and Smith (2).

TABLE I —TAIL FLICK REACTION TIME OF RATS AT 48 AND 50°

Time of Stimulus, min.	Reaction Time, sec.[a] 48°	50°
0	4.8(1.9)[b]	2.9(0.9)
15	5.4(1.5)	3.4(1.2)
30	5.4(1.7)	3.5(1.5)
Mean	5.2(1.7)	3.3(1.3)

[a] Average of 31 animals. [b] Standard deviation in parentheses.

TABLE II —TAIL FLICK REACTION TIME OF RATS AT 48 AND 50° AFTER REPEATED EXPOSURE TO STIMULUS FOR THREE WEEKS

Time of Stimulus, min.	Reaction Time, sec.[a] 48°	50°
0	5.6(1.5)[b]	3.3(0.7)
15	5.4(1.9)	3.5(1.0)
30	5.5(1.3)	3.9(1.1)
Mean	5.5(1.6)	3.6(1.0)

[a] Average of 21 animals. [b] Standard deviation in parentheses.

The animals used in this investigation neither were especially selected for their normal response, nor were trained in any special manner. According to some investigators (3–5), preselection and training of the experimental animals yields more consistent data, but even without these precautions it was possible in this study to demonstrate the effect of small temperature differences on reaction time. The results show that the temperature of the heat source used for thermal stimulation must be closely controlled if the sensitivity and reproducibility of thermal analgesimetric methods is to be assured.

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ERRATUM

In the paper titled "Toxic Saponin from *Elvira biflora*" (1), qualitative test result (*f*), page 780, column 2, should read (*f*) the same water solution boiled with cholesterol did not hemolyze blood cells of the guinea pig in phosphate buffer.

(1) de Oliveira, M. M., and Andrade. S. O., THIS JOURNAL, **50**, 780(1961).

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[a] Average of 31 animals. [b] Standard deviation in parentheses.

TABLE II —TAIL FLICK REACTION TIME OF RATS AT 48 AND 50° AFTER REPEATED EXPOSURE TO STIMULUS FOR THREE WEEKS

Time of Stimulus, min.	Reaction Time, sec.[a] 48°	50°
0	5.6(1.5)[b]	3.3(0.7)
15	5.4(1.9)	3.5(1.0)
30	5.5(1.3)	3.9(1.1)
Mean	5.5(1.6)	3.6(1.0)

[a] Average of 21 animals. [b] Standard deviation in parentheses.

The animals used in this investigation neither were especially selected for their normal response, nor were trained in any special manner. According to some investigators (3–5), preselection and training of the experimental animals yields more consistent data, but even without these precautions it was possible in this study to demonstrate the effect of small temperature differences on reaction time. The results show that the temperature of the heat source used for thermal stimulation must be closely controlled if the sensitivity and reproducibility of thermal analgesimetric methods is to be assured.

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ERRATUM

In the paper titled "Toxic Saponin from *Elvira biflora*" (1), qualitative test result (*f*), page 780, column 2, should read (*f*) the same water solution boiled with cholesterol did not hemolyze blood cells of the guinea pig in phosphate buffer.

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Colorimetric Method for the Determination of Vinblastine, an Alkaloid from *Vinca rosea* I

By IVAN M. JAKOVLJEVIC

A reproducible colorimetric method is described for the determination of vinblastine, an antileukemic alkaloid from *Vinca rosea* Linn.

VINBLASTINE, an alkaloid from the ornamental shrub *Vinca rosea* Linn. was first reported as vincaleucoblastine[1] by Noble, Beer, and Cutts (1). On the basis of analytical and physical data the empirical formula of $C_{46}H_{58}N_4O_9$ (2) has been suggested for vinblastine. Spectral studies of this and parent alkaloids, catharanthine, and vindoline indicated that vinblastine represented a new type of dimeric alkaloid containing indole and indoline moieties (3). Vinblastine has been shown to contain the following functional groups: 2-$COOCH_3$, $OCOCH_3$, OCH_3 (aromatic), OH (free), OH (hydrogen bonded), NH (indole), and NCH_3. The appropriate derivatives of vinblastine as well as functional group analysis corroborate the presence of these groups (4).

Since some 20 different alkaloids have been obtained from this plant (5), a specific method for the detection and determination of vinblastine and its salts were highly desirable. The alkaloid produces color reactions with several reagents. If about 1 mg. of the alkaloid is heated with 50% hydrochloric acid containing 0.2% vanillin, a pink color is produced. With concentrated sulfuric acid containing traces of formaldehyde, a light wine color results. Concentrated sulfuric acid gives no color at room temperature; nitric acid gives a yellow color. A faint pink color is obtained with the alkaloid when heated with 40–60% sulfuric acid. Ten per cent sulfuric acid containing 1% of cerium sulfate gives a dark orange color which rapidly changes to yellow. A dark blue color is obtained by dissolving vinblastine in a 1% solution of ferric ammonium sulfate in 75% sulfuric acid. If vinblastine is dissolved in formic acid and several drops of 5% sodium hypochlorite solution are added, an orange color develops which fades to yellow.

After experimentation with many reagents, it was found that addition of vinblastine to an appropriate mixture of acetic anhydride, acetyl chloride, pyridine, and sulfuric acid followed by heating, produced a deep rose color. This color development is specific for vinblastine and, up to now, has not been observed with any other alkaloid reported from *V. rosea*. The specificity of this reaction has been corroborated qualitatively by paper chromatography of the alkaloids from *V. rosea* (6).

This paper describes the details for the colorimetric determination of vinblastine.

EXPERIMENTAL

Moisture Determination.—Vinblastine sulfate contains moisture which can be removed by vacuum drying at about 50° without damage to the alkaloid. The anhydrous material, however, is very hygroscopic and picks up about 2% moisture in 2 minutes when allowed to stand under room conditions. Equilibrium is reached in about 20 minutes, resulting in a moisture content of about 10%. The most convenient method of handling the compound is to allow it to reach equilibrium, at which time separate samples are weighed for the color assay and for the moisture determination. The moisture is determined by vacuum drying at 50° for 16 hours.

Apparatus and Reagents.—A Beckman model DU spectrophotometer with 1-cm. glass cells was used in this laboratory, but any suitable spectrophotometer may be used.

A constant temperature oil bath was used to insure the exclusion of condensation water during the heating period. The oil bath used was designed to hold eight 10-ml. volumetric flasks so that a standard and three samples, in duplicate, could be run simultaneously.

Care must be taken to insure the exclusion of water from the reagents and glassware.

Acetyl chloride, analytical reagent grade.

Acetic anhydride, analytical reagent grade. This reagent must contain a minimum of 97% $(CH_3CO)_2O$, and must pass the U.S.P. test for reducing substances. At least 24 hours before use, 0.25 ml. of acetyl chloride is added to each 500 ml. of acetic anhydride. The activated acetic anhydride can be stored for at least 3 months. The metallic lining of the acetic anhydride bottle cap should be replaced with an inert plastic material. A corroded metallic liner may indicate an undesirable reaction which reduces the color formation.

Pyridine, analytical reagent grade. Water content must be less than 0.1%.

Sulfuric acid, concentrated, analytical reagent grade.

Preparation of the Color Reagent.—The color reagent must be freshly prepared immediately prior to use as indicated in the procedure.

By means of a graduate, 35 ml. of acetic anhydride is transferred to a 100-ml. glass-stoppered Erlenmeyer flask and cooled under running water. While swirling and cooling, 1 ml. of concentrated sulfuric acid is added. With continued cooling and swirling, 35 ml. of pyridine is added in about three equal portions.

Procedure.—About 3 mg. of standard vinblastine sulfate, and about 3 mg. of the sample preparation are weighed accurately to the nearest 0.01 mg. Each is transferred quantitatively to separate 10-ml. volumetric flasks. From this point on, the assay should be performed without delay. The

Received April 10, 1961, from the Analytical Development Laboratory, Eli Lilly and Co., Indianapolis, Ind.

Accepted for publication May 2, 1961.

The author wishes to express his indebtedness to Mr. Norbert Kuzel and Mr. LeRoy Springman for their suggestions, and to Dr. Norbert Neuss for helpful discussions concerning this paper. The author wishes to thank Mr. William Beach for the design of special equipment used, and Mr. William Shaffer for his assistance in the collection of the large amount of data necessary for the development of this method.

[1] In compliance with the A.M.A. Council on New Drugs, its name has been changed to vinblastine. The sulfate salt of this alkaloid under the name of Velban (vinblastine sulfate, Lilly) has recently been made available for the treatment of Hodgkin's disease and choriocarcinoma.

standard and sample are dissolved and diluted to the mark with the color reagent and well mixed. Two 2-ml. aliquots of the standard solution are transferred to separate 10-ml. volumetric flasks labeled "standard." Two 2-ml. aliquots of the sample solution are transferred to separate 10-ml. volumetric flasks labeled "sample." To each of the four flasks 3 ml. of the color reagent is added by pipet. The flasks are placed simultaneously in an oil bath which has been preheated to 80 ± 1°. After about 2 minutes of heating, the flasks are stoppered loosely with glass stoppers. The flasks are heated a total of exactly 20 minutes at 80 ± 1°, then removed from the bath and cooled to room temperature under running water. Each flask is diluted to the mark with color reagent and well mixed. The absorbance of each is measured on a suitable spectrophotometer in 1-ml. cells at 574 and 538 mμ, using distilled water as a reference.

The ratio A_{574}/A_{538} is determined. This value must be within the range of 1.20 to 1.25 for relatively pure vinblastine. Any value outside this range indicates the color has been incorrectly developed. In the case of crude alkaloid mixtures containing less than 30% vinblastine, the ratio may be slightly less than 1.20.

Calculations.—

$$\frac{\text{Abs.}_{574}\ \text{sample} \times \text{mg. standard}}{\text{Abs.}_{574}\ \text{standard} \times \text{mg. sample}} \times 100 = \%\ \text{vinblastine sulfate}$$

$$\frac{\%\ \text{vinblastine sulfate}}{1.000 - \frac{\%\ \text{loss of wt. on drying}}{100}} = \%\ \text{vinblastine sulfate, anhydrous}$$

DISCUSSION

Standard Curve.—A plot of the absorbance *versus* concentration was found to be linear and to pass through the origin, obeying Beer's law from 5–70 mcg. per ml. (Fig. 1). The reproducibility of the method is entirely satisfactory and will be discussed in a later paper.

Stability of the Color.—The color is stable for about 20 minutes after which time it begins to increase slowly at 574 mμ, and more rapidly at 538 mμ (Fig. 2). This differential rate of increase of the color at the two wavelengths was responsible for the choice of 574 mμ as the wavelength for quantitative measurements.

Influence of Sulfuric Acid.—One milliliter of sulfuric acid seems to be the optimum amount to add to the 70 ml. of 1:1 acetic anhydride:pyridine mixture. The addition of more or less sulfuric acid reduces the color intensity and conformity to Beer's law is reduced to a much narrower range of concentrations.

Influence of Acetyl Chloride.—Acetyl chloride has an important influence on the color development. Without acetyl chloride, the color develops a pronounced yellow tint and the absorption peak at 574 mμ is shifted to lower wavelengths. With a few lots of acetic anhydride it was not necessary to add acetyl chloride. In these cases it is supposed that trace amounts of acetyl chloride were present due to the method of synthesis of the anhydride.

Characterization of the Color Produced.—It is possible to isolate a deep rose oil from the reaction mixture after the color has been developed. The excess reagent was removed from the mixture by several washings and decantations with large amounts of ether. The oily residue rapidly changed to a bright brown color. When color reagent was added to the residue, the original rose color was reconstituted; however, the ratio A_{574}/A_{538} was slightly diminished.

The chemistry of the color formation is not clear at the present time. There is a possibility that the color is due to a halochromic effect of the reaction mixture. Clarification on this point may result from further elucidation of the structure of this alkaloid, presently being conducted in these laboratories.

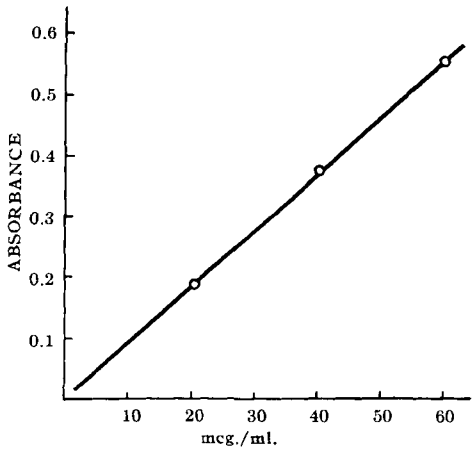


Fig. 1.—Calibration curve of vinblastine sulfate.

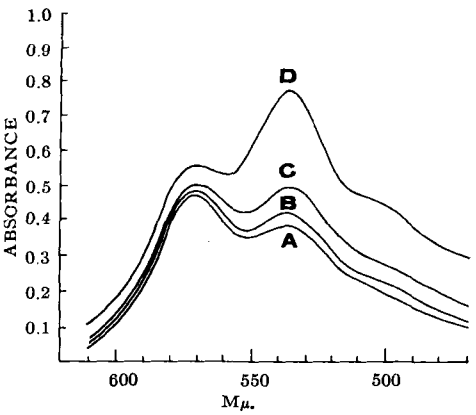


Fig. 2.—Cary recording spectrophotometric curves of the color. A, Immediately after diluting to volume; B, 1 hour after diluting to volume; C, 2 hours after diluting to volume; D, 4 hours after diluting to volume.

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Tetrazole Analogs of Various Amino Acids as Possible Bacterial Antagonists

By W. A. ZYGMUNT

RECENTLY, the synthesis of the tetrazole analogs of several amino acids was described by McManus and Herbst (1). Since we had an opportunity to obtain several of these compounds,[1] it was of interest to determine whether they had any activity as amino acid antagonists in bacteria.

The tetrazole analogs tested were as follows: I, 5-aminomethyltetrazole (glycine analog); II, 5-(1′-aminoethyl)tetrazole (DL-alanine analog); III, 5-(2′-aminoethyl)tetrazole (β-alanine analog); IV, 5-(1′-aminopropyl)tetrazole (DL-α-aminobutyric acid analog); and V, 5-(1′-amino-2′-phenylethyl)tetrazole (DL-phenylalanine analog).

Each of the compounds was tested with a microorganism which synthesizes its own amino acids, and certain compounds with microorganisms which require specific amino acids for growth. All compounds were tested for their ability to inhibit the growth of *Escherichia coli* W in the synthetic medium of Davis and Mingioli (2).

In addition, test compounds I and V were tested for their ability to inhibit utilization of glycine and phenylalanine in the amino acid basal medium of Barton-Wright (3) using *Leuconostoc mesenteroides* P60, an organism requiring these amino acids. Compound II was also tested for antagonism of DL-alanine utilization in the medium of Sauberlich and Baumann (4) using *Pediococcus cerevisiae* (ATCC No. 8081), an alanine-dependent organism. Neutralized solutions of test compounds, amino acids, and basal media were sterilized by Seitz filtration and portions added to previously autoclaved culture tubes containing graded levels of sterile, distilled water. Only freshly prepared solutions of the test compounds were used. Final assay volumes were 6 ml. per culture tube (18 × 150 mm.). In the preparation of the inocula, precautions were taken to minimize the carry-over of preformed amino acids by appropriate washing of the cultures and by the use of small inocula. Incubation periods of 24 hours were used with *E. coli* and *L. mesenteroides*, and 72 hours with *P. cerevisiae*. An incubation temperature of 35° was used with all cultures. Growth was measured turbidimetrically as absorbance using a Coleman junior spectrophotometer at a wavelength of 620 mμ.

All of the tetrazole analogs studied, at a final concentration of 1 mg. of test compound per ml. (molar range of 0.005 to 0.010), failed to inhibit the growth of *E. coli* in a 24-hour assay. This clearly indicates that these compounds (I through V) are not potent inhibitors of the synthesis and/or utilization of amino acids.

TABLE I.—ANTAGONISM OF DL-ALANINE UTILIZATION BY 5-(1′-AMINOETHYL)TETRAZOLE IN *P. cerevisiae*

Molarity 5-(1′-Aminoethyl)-tetrazole	Molarity DL-Alanine	Molar Ratio Antagonist to Metabolite	% Inhibition of Growth, 72 hours at 35°
0.022	0.000093	237/1	98
0.022	0.000186	118/1	38
0.022	0.000372	59/1	19
0.040	0.000093	430/1	99
0.040	0.000186	215/1	50
0.040	0.000372	108/1	10
0.040	0.000744	54/1	4
0.040	0.001488	27/1	3

Compound I at a 0.04 *M* concentration when tested with *L. mesenteroides* in the presence of 0.0004 *M* glycine (half maximal growth) inhibited the growth of *L. mesenteroides* by 28%. No inhibition of growth was observed at 0.005 *M*, 0.01 *M*, and 0.02 *M* concentrations. This low order of activity was not sufficient to warrant further studies.

In the presence of 0.0002 *M* DL-phenylalanine, the level required for half maximal growth, no inhibition of growth was observed at 0.05 *M* and 0.01 *M* concentrations of compound V. It was not possible to test higher concentrations due to the low solubility of this compound at a neutral pH.

5-(1′-Aminoethyl)tetrazole (compound II) was shown to be an antagonist of DL-alanine utilization with *P. cerevisiae*. This anatagonism was readily reversed by DL-alanine. The compound must be considered a weak inhibitor, however, in that approximately a 200–400 to 1 molar ratio of antagonist to metabolite was needed to obtain a 50 to 99% inhibition of growth (Table I).

Further tests on compounds I, II, and V with *L. mesenteroides* and *P. cervisiae* which require glycine, phenylalanine, or α-alanine, respectively, showed that the analogs did not support growth in place of the respective amino acids.

In summary, tetrazole analogs of five different amino acids were found to have little or no activity as inhibitors of bacterial growth in chemically defined media. As yet, none of the available tetrazole analogs have been studied in isolated enzyme systems.

Received August 14, 1961, from the Department of Nutritional and Biochemical Research, Mead Johnson Research Center, Evansville 21, Ind.

Accepted for publication November 8, 1961.

The author gratefully acknowledges the valuable advice of Drs. Herbert P. Sarett and Homer E. Stavely in these studies, and the test compounds kindly supplied by Dr. Robert M. Herbst.

[1] Obtained from Dr. R. M. Herbst, Department of Chemistry, Michigan State University, East Lansing, Mich.

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Progress in Drug Research. Vol. 2. Edited by ERNST JUCKER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960, 636 pp. 16.5 × 24 cm. Price $22.50

The development of a reference work in the field of drug research is continued with the issuance of volume 2 in this series. The publication of volume 1 was noted in THIS JOURNAL, 49, 62(1960). Other volumes will appear annually. Extensive review articles cover nine subjects in volume 2. The articles are printed in the languages of the contributors. In this volume five articles are in English and four are in German. Indexes are not appended, but each article has a detailed table of contents.

Investigation of Rates and Mechanisms of Reactions. Part 1. 2nd ed. Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1961. xii + 713 pp. 15.5 × 22.5 cm. Price $23.50.

A revised and augmented edition of volume 8, part 1, in the series "Technique of Organic Chemistry." The first edition was reviewed in THIS JOURNAL, 42, 507(1953). The text should be extremely useful in the field of physical pharmacy. An index is appended.

Metabolic Pathways in Microorganisms. By VERNON H. CHELDELIN. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1961. ix + 91 pp. 12.5 × 18.5 cm.

A compilation of lectures on carbohydrate metabolism in microbial systems, emphasizing metabolic peculiarities of acetic acid bacteria.

Book Notices

Virus Meningo-Encephaltis. Edited by G. E. W. WOLSTENHOLME and MARGARET P. CAMERON. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1961. viii + 120 pp. 12.5 × 18.5 cm.

A compilation of 13 papers and discussions on subjects related to meningo-encephalitis which were presented at a study group in September 1960. References and a subject index are appended.

Quinones in Electron Transport. Edited by G. E. W. WOLSTENHOLME and CECILIA M. O'CONNOR Little, Brown and Co., 34 Beacon Street, Boston 6, Mass., 1961 xii + 453 pp. 13.5 × 20.5 cm. Price $11.

Reports on biochemical studies related to the quinones, particularly ubiquinone (coenzyme Q), and their part in electron transport are compiled in this book. Nineteen papers were contributed and discussed at the May 1960 Ciba Foundation Symposium.

Pharmacology and Oral Therapeutics. 12th ed. By EDWARD C. DOBBS. The C. V. Mosby Co., 3207 Washington Blvd., St. Louis 3, Mo., 1961. 578 pp. 16 × 24 cm. Price $10.

Revision of this authoritative textbook assures consideration of those drugs official in U.S.P. XVI and N.F. XI, as well as the drugs evaluated in the latest editions of "New and Nonofficial Drugs" and "Accepted Dental Remedies." The book is excellent for dental students and practitioners, and includes useful information for pharmacists.

Comparative Animal Physiology. 2nd ed. By C. LADD PROSSER and FRANK A. BROWN, JR. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1961. ix + 688 pp. 16.5 × 25.5 cm. Price $15.50.

Intended as a text for upper-class undergraduates and first-year graduate students and as a reference volume, this edition reflects the additional knowledge and current views developed since the 1950 edition. The authors consider the reactions of animals to specific variations in a series of environmental factors and then consider effector and coordinating mechanisms in general.

Biochemists' Handbook. Edited by CYRIL LONG. D. Van Nostrand Co., Inc., 120 Alexander St., Princeton, N. J., 1961. xxii + 1192 pp. 15.5 × 25 cm. Price $25.

Compiled chiefly for biochemists and others in related sciences, this encyclopedia-type handbook is divided into six sections. Section 1 includes selected topics in physical and organic chemistry, with tabulated data. Section 2 discusses enzyme kinetics and describes about 300 enzymes. Metabolic pathways are treated in Section 3. Data on the chemical composition of animal tissues and plants are given in Sections 4 and 5, respectively. Section 6 gives information on some physiological and nutritional topics. The general index requires 64 pages.

Physical Chemistry of Macromolecules. By CHARLES TANFORD. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1961. xiv + 710 pp. 15 × 23 cm. Price $18.

Areas of physical chemistry in this book are taken up in the order: Molecular structure, with emphasis on X-ray diffraction; Statistics of linear polymers; Thermodynamics; Light scattering; Transport processes and viscosity; Electrostatic free energy and polyelectrolytes; Multiple equilibria; and Kinetics of macromolecular reactions. Fundamental theories are given and then modified or extended to apply to molecules of high molecular weight. Author and subject indexes are appended

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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Review Article

Structure-Activity Relationships of Drugs Affecting the Lungs

By DOMINGO M. AVIADO

THE LUNGS can respond to drugs in four different ways: (*a*) there are sensory nerve endings in the lung parenchyma which can be stimulated chemically to influence respiration, (*b*) the smooth muscles of the bronchioles can be contracted or relaxed by drugs and therefore alter the resistance to flow of air during respiration, (*c*) the smooth muscles of the pulmonary vessels can be constricted or dilated by drugs and therefore alter the resistance and pressure of blood flowing in the pulmonary circulation, and (*d*) the total amount of pulmonary blood flow can be reduced or increased by a primary action of drugs on the heart and systemic vessels. This paper will review each of these four types of actions with special emphasis on the relationships of chemical structure to physiological activity as observed on various structures of the lungs.

It should be stated at the outset that no drug has its action limited to only one structure in the lungs. For example, one sympathomimetic amine, like epinephrine, can influence bronchiolar muscle tone, pulmonary vascular tone, and pulmonary blood flow. Another sympathomimetic amine, such as methoxamine, can influence only pulmonary blood flow, but not directly the bronchiolar and pulmonary vascular smooth muscles; however, the consequences of a primary systemic pressor action include reflex increase in vagal tone which can include bronchoconstriction. These examples serve to demonstrate the differences in extent of action of drugs belonging to the sympathomimetic class, and this is true also for xanthines and antihistaminics which will be discussed below. An attempt will be made to explain such differences on the basis of chemical structure. As new compounds are developed, it is possible that some of them may prove clinically useful as selective respiratory stimulants, bronchodilators, and pulmonary vasodilators.

REFLEXES FROM THE LUNGS

Nature of Drug Action.—The respiratory effects of chemical stimulation of receptors in the lungs have been reviewed (1, 2). These receptors are supplied by the vagal innervation of the lungs and, when stimulated, would cause temporary apnea followed by tachypnea. These receptors that influence respiration are believed to be normally functioning as stretch receptors in the lung parenchyma (Hering-Breuer reflex) and baroreceptors in the pulmonary vessels (Bezold-Jarisch reflex). It has not been possible to show any direct response to the latter receptors because isolation from other vagal receptors innervating surrounding structures is difficult.

Received from the Department of Pharmacology, Schools of Medicine, University of Pennsylvania, Philadelphia 4.

The experiments discussed herein were supported by the U. S. Army Medical Branch and Development Command, Department of the Army, under Contract No. DA-49-193-MD-2093.

Adapted from a paper presented at the Gordon Research Conference on Medicinal Chemistry, Colby Junior College, New London, N. H., July 31 to August 4, 1961.

Active Compounds.—The following compounds have been shown to increase the activity of the pulmonary stretch receptors: inhalation of ether, trichlorethylene, and chloroform (3); intravenous injection of veratrum alkaloids (4–6), 5-hydroxytryptamine (7), amidines (8), aconitine (9), strychnine (10), and antihistaminic compounds (11). The following compounds have been shown to elicit the reflex from the lungs, but action potential studies are still lacking: nicotine (12), lobeline (13), tributyrin (14), capsaicin (15, 16), andromedotoxin (17), benzofurans (18), and aminoethylisothiuronium (19).

The variety of compounds that elicit a respiratory reflex from the lungs makes it difficult to specify a common pharmacological action to explain the response. It is very probable that the receptors are stimulated in one of three ways: (*a*) direct action of the compound on the sensory nerve ending; (*b*) direct action of the compound on the vessels which will in turn stimulate the baroreceptors; and (*c*) direct action of the compound on the bronchiolar smooth muscle which will in turn influence the sensitivity of the stretch receptors. Although actions (*b*) and (*c*) will be discussed below, it should be stated here that such actions have not been investigated for most of the compounds that elicit the respiratory reflex.

Antihistaminic Compounds.—This group deserves some special attention because it has been adequately studied from several standpoints. Winder and Thomas (20) reported that diphenhydramine caused apnea followed by tachypnea when injected intravenously in the anesthetized dog. Because the effects were eliminated by vagotomy, they postulated that the respiratory effect was due to a central augmentation by the drug of normal vagal proprioceptive impulses from the lungs. Catheterization of the cardiopulmonary circulation showed that this antihistaminic drug was acting not centrally, but peripherally, on receptors in the lungs (21). Takasaki, *et al.* (22), reconfirmed the reflex nature of the response in other animal species (cat and rabbit). Jones (23) showed in such species that the respiratory reflex could not be explained entirely by pulmonary stretch receptors but suggested other receptors in the lungs which have not yet been identified. Unlike most other compounds, the antihistaminics do not stimulate cardiac receptors responsible for bradycardia and hypotension.

Possible Clinical Use.—In the anesthetized animal, the reflex induced by antihistaminic compounds consists of an initial apnea followed by prolonged tachypnea. If the latter response were confirmed in man, it would be possible to develop a new type of respiratory stimulant. Most clinically useful stimulants act centrally and therefore have other undesirable effects (convulsions), so that a stimulant acting exclusively by reflexes would be expected to be free of such central actions.

Structure-Activity Relationships.—Another special feature of the antihistaminic group is that 27 compounds have been tested for reflex respiratory action (21). The results allow the following generalizations in structure-activity relationships: (*a*) Almost all of the antihistaminic compounds tested have a basic ethylamine structure. (*b*) The nature of substitution of the amine partly determines the ability of the compound to elicit the reflex. The most active compounds have either two methyl or two ethyl substituents. (*c*) The nature of aromatic substituents at the β-carbon atom also influences the activity of the compound. The active and inactive substituents are summarized in Fig. 1.

BRONCHODILATATION

Active Compounds.—Hawkins (24) has compiled a series of tables which summarize the effects of several types of compounds on the bronchiolar smooth muscles. It is apparent from such tables that several groups of compounds can be depended upon to relax bronchiolar smooth muscles, particularly after spasm induced by histamine. The groups are as follows: sympathomimetic amines, parasympathetic blocking drugs, local anesthetics, antihistaminics, and a miscellaneous group of compounds with musculotropic action.

Sympathomimetic Amines.—This group has been used widely in the treatment of bronchial asthma. The major limitation in the clinical use is the accompanying effects on the cardiovascular system. These side effects have been systematically compared in anesthetized dogs (25), and the pattern of action for each of six bronchodilators are as follows: (*a*) Isoproterenol injected directly into the common carotid, vertebral, superior mesenteric, and external iliac arteries causes vasodilatation. This action is the major cause for the systemic arterial hypotension. It masks the accompanying cardiac stimulation which can be detected as increases in myocardial force of contraction and in pulmonary blood flow. (*b*) Methoxyphenamine and (*c*) isoprophenamine cause systemic vasodilatation similar to that of isoproterenol, but much larger doses are required. The accompanying effect on the heart is a depression of myocardial force of contraction. (*d*) Epinephrine causes vasoconstriction when injected into

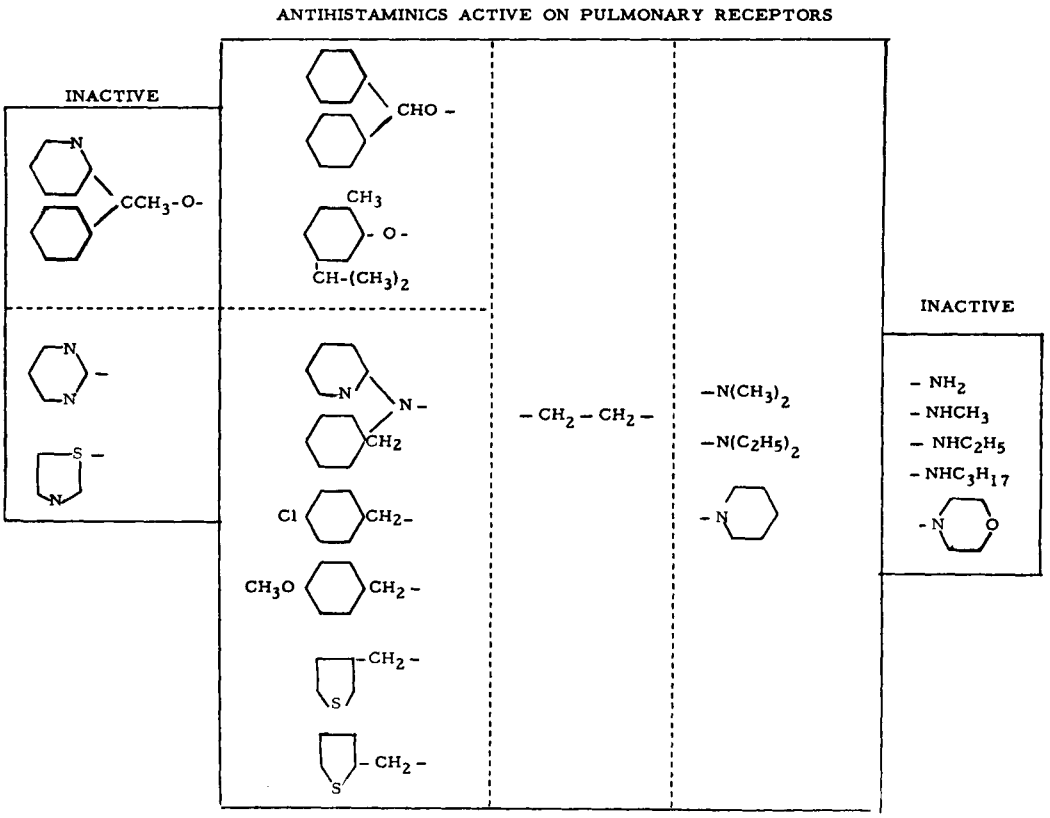


Fig. 1.—Active and inactive substituents associated with stimulation of pulmonary reflexes by antihistaminics.

Sympathomimetic Drugs	Phenyl		Ethyl		Amine	Cardiac stimulant	Vaso-constriction	Vaso-dilatation	Broncho-dilatation
1) Epinephrine	3-OH	4-OH	OH		CH_3	+	+	+	+
2) Levarterenol	3-OH	4-OH	OH			+	+	–	–
3) Isoproterenol	3-OH	4-OH	OH		C_3H_7	+	–	+	+
Cardiac stimulant						+	–	–	–
Bronchodilator						–	–	–	+
4) Metaraminol	3-OH		OH	CH_3		+	+	–	–
5) Phenylephrine	3-OH		OH		CH_3	–	+	–	–
6) Hydroxyamphetamine		4-OH		CH_3		+	+	–	–
7) Nylidrin		4-OH	OH	CH_3	$C_{10}H_{13}$	+	–	+	+
Cardiac stimulant						+	–	–	–
Vasodilator						–	–	+	–
Bronchodilator						–	–	–	+
8) Methoxamine	2-CH_3O	5-CH_3O	OH	CH_3		–	+	–	–
9) B. W. 45-50	2-C_2H_5O	5-C_2H_5O	OH	CH_3		–	+	+	–
10) Methoxyphenamine	2-CH_3O			CH_3		–	–	+	+
11) Isoprophenamine	2-Cl		OH		C_3H_7	–	–	+	+
Vasodilator						–	–	+	–
Bronchodilator						–	–	–	+
12) Ephedrine			OH	CH_3	CH_3	+	+	–	+
13) Methamphetamine				CH_3	CH_3	+	–	+	–
14) Mephentermine				$(CH_3)_2$	CH_3	+	–	+	–
Cardiac stimulant						+	–	–	–
Vasodilator						–	–	+	–
Bronchodilator						–	–	–	+

Fig. 2.—Comparison of chemical structure and side effects of sympathomimetic bronchodilators.

the various systemic arteries. The systemic hypertensive response following its intravenous injection is due both to vasoconstriction and to stimulation of myocardial force of contraction. (*e*) Ephedrine causes vasoconstriction in the various systemic arterial beds and stimulation or depression of myocardial force. (*f*) Pseudoephedrine is a weaker constrictor than ephedrine for the common carotid, external iliac, and superior mesenteric arteries. Unlike ephedrine, pseudoephedrine causes vasodilatation in the area supplied by the vertebral artery. The stimulation and depression of myocardial contraction for both ephedrine and pseudoephedrine are equal in intensity.

Structure-Activity Relationships.—A comparison of the chemical structure of bronchodilator amines has failed to make a distinction between active and inactive substituents (Fig. 2). The 3,4-dihydroxy substitutions common to both epinephrine and isoproterenol are lacking in ephedrine and pseudoephedrine; methoxyphenamine has a 2-methoxy group whereas isoprophenamine contains a 2-chloro substituent in the phenyl ring. The attachment of an hydroxyl or a methyl radical in the ethyl chain is not a normal feature of all bronchodilators. The only common feature is a substitution in the amine and this is true for other sympathomimetic amines that have been proven effective in experimental animals (24). The need for new bronchodilators appears largely to be for a more selective one with minimal activity on the cardiovascular system; the structure-activity relationships for bronchodilatation should be correlated with those for cardiovascular actions discussed below.

PULMONARY VASODILATATION

Types of Vasodilators.—The literature on the effects of drugs on the pulmonary vessels has been reviewed recently (26). The vasodilators that have been used clinically to reduce pulmonary hypertension include aminophylline, isoproterenol, and tolazoline. Although each of these three agents is able to dilate the pulmonary vessels, they also dilate the peripheral vessels, and stimulate the heart. The latter action is accompanied usually by an increase in cardiac output or pulmonary blood flow so that the net result of the drug may even be a rise in pulmonary arterial pressure. Derivatives of aminophylline have been examined and these will be discussed next.

Structure-Activity Relationships of Xanthines.—Forty derivatives of aminophylline (theophylline ethylenediamine) were tested in anesthetized dogs to compare the vascular action in the lungs and the extremities (27). All compounds tested revealed either dilatation for both, or constriction for both, but never opposite effects (Fig. 3).

Theophylline (1,3-dimethylxanthine) gave essentially the same results as those described for aminophylline (theophylline ethylenediamine); i.e., pulmonary and femoral vasodilatation. Caffeine (1,3,7-trimethylxanthine) was slightly more powerful than aminophylline in dilating the pulmonary vessels but weaker in dilating the femoral vessels. Theobromine (3,7-dimethylxanthine) was half as active as theophylline in dilating the vessels of the lungs and legs. The following generalizations apply to the correlation of chemical structure of the other xanthines tested for their vascular effect: (*a*) unsubstituted xanthine is a weak dilator (less than one-half the potency of theophylline) for the lung and limb vessels; (*b*) replacement of the oxygen linked to the carbon atoms at positions 2 and/or 6 with sulfur does not affect the dilator action; (*c*) lengthening the carbon chain attached to the nitrogen at position 3 does not alter the dilator effect characteristic of caffeine, theobromine, and theophylline; (*d*) lengthening the carbon chain attached to the nitrogen at position 1 beyond one carbon converts the dilator action of theophylline and caffeine to constriction; (*e*) lengthening the carbon chain at No. 7 nitrogen position beyond the methyl substituent converts the dilator action of theobromine to vasoconstriction; however, if there is an hydroxyl or chlorine attached to the ethyl substituent, the dilator action is restored; (*f*) an additional substitution at No. 8 carbon position either maintains or reverses the dilator action of theophylline or caffeine. Lengthening the carbon chain beyond a methyl group converts the compound to a vasoconstrictor. However, the benzyl and the *p*-aminobenzyl substituted compounds retain their vasodilator effects. Hydroxyl or amino substitution of the methyl carbon results in a vasoconstrictor, as is the carbamyl substituted methylamino compound. An aliphatic or aromatic ether group, and the nitro and sulfhydryl analogs, also give rise to vasoconstrictors. The 4-methylpiperazinyl analog is a vasodilator, whereas the amino substituted compound is a vasoconstrictor.

Patterns of Responses of Sympathomimetic Amines.—Eighty amines have been tested in the anesthetized dog for effects on pulmonary vascular tone and pulmonary blood flow (28). The results of 22 of them which are commercially available are summarized in Fig. 4. It can be

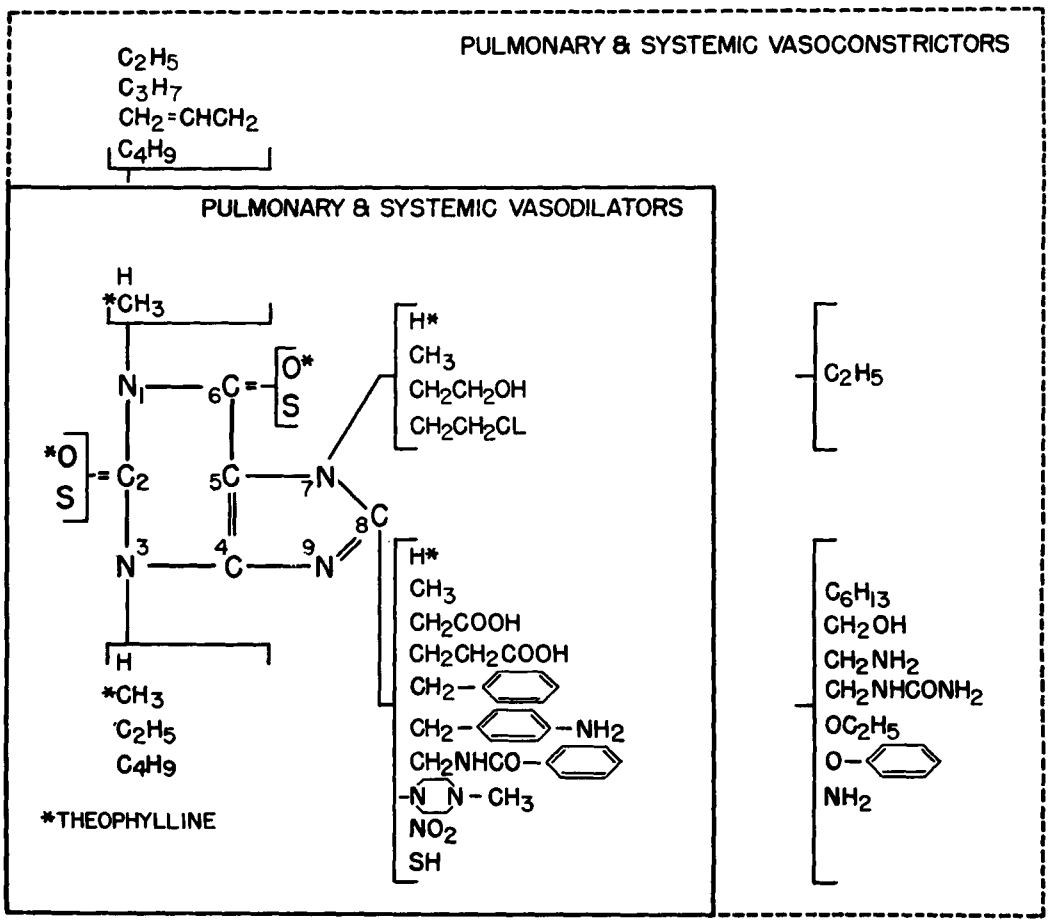


Fig. 3.—Chemical structures of xanthines which cause nonselective vasodilatation or vasoconstriction. Reprinted from reference 27 with the permission of the publishers.

noted that the effects on vessel tone or flow can be grouped into increase, decrease, or variable effect so that there are nine possible combinations of actions. Among the pulmonary vasodilators, the diethoxyl derivative of methoxamine (compounds 45–50) can consistently cause a fall in pulmonary blood pressure because the pulmonary blood flow is reduced. The reduction in flow appears to be related to the substitutions in the 2 and 5 positions of the phenyl ring.

PULMONARY BLOOD FLOW

In the above discussion of sympathomimetic dilators for the lung vessels it was stated that hemodynamically, a reduction in pulmonary arterial pressure is more likely to occur if the drug reduces pulmonary blood flow or cardiac output. An exception to this would be a relative failure of the left ventricle so that the reduction in output would mean a rise in left atrial pressure, pulmonary venous pressure, and even pulmonary arterial pressure. It is for this reason that the sympathomimetic amines were tested in an attempt to find a pulmonary vasodilator with no reduction of pulmonary blood flow, but with less conspicuous increase in pulmonary blood flow than with isoproterenol and mephentermine. Seven derivatives of mephentermine were tested and the results are reported below.

Methods.—Dogs under morphine (2 mg./Kg., s.c.) and chloralose (70 mg./Kg., i.v.) anesthesia were used. The following procedures were routinely performed in all animals: (*a*) cannulation of the trachea to allow the use of a Starling Ideal pump, (*b*) cannulation of a femoral vein for drug injection, (*c*) catheterization of a carotid artery for recording of aortic blood pressure by a Statham transducer, and (*d*) opening of the chest in the left fifth intercostal space to allow measurements of pressure in the pulmonary artery from a catheter tied into the artery of the left upper lobe. The additional procedures performed in each of two groups of dogs consisted of the following: (*e*) measurement of

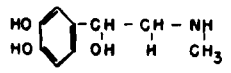
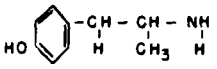
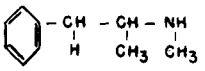
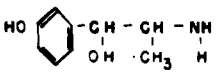
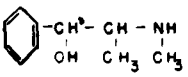
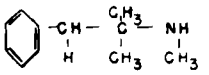
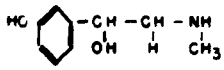
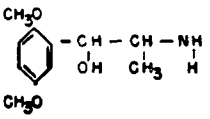
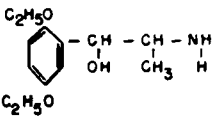
	PULMONARY VASOCONSTRICTOR	VARIABLE OR NONE	PULMONARY VASODILATOR
INCREASED PBF	EPINEPHRINE  LEVARTERENOL (LEVOPHED) TUAMINOHEPTANE (TUAMINE) EPININE TYRAMINE	HYDROXYAMPHETAMINE (PAREDRINE)  PHOLEDRINE (PAREDRINOL) SYNEPHRIN (SYMPATOL)	METHAMPHETAMINE (METHEDRINE)  ISOPROTERENOL (ISUPREL) NYLIDRIN (ARLIDIN)
VARIABLE OR NONE	METARAMINOL (ARAMINE) 	EPHEDRINE  AMPHETAMINE (BENZEDRINE) PHENYLPROPANOLAMINE (PROPADRINE) METHYLAMINOHEPTANE (OENETHYL) NAPHAZOLINE (PRIVINE)	MEPHENTERMINE (WYAMINE)  PHENYLPROPYLMETHYLAMINE (VONEDRINE) CYCLOPENTAMINE (CLOPANE)
DECREASED PBF	PHENYLEPHRINE (NEOSYNEPHRINE) 	METHOXAMINE (VASOXYL) 	B. W. COMPOUND 45-50 

Fig. 4.—Chemical structure of sympathomimetic amines and their effect on pulmonary circulation.

pulmonary venous outflow (28) and force of myocardial contraction by means of a strain gauge arch sutured to the surface of the right ventricle (29), (*f*) inflow perfusion of the left lower lobe (28) combined with measurement of femoral arterial blood flow by insertion of a Shipley-Wilson recording rotameter. All these methods are depicted in Fig. 5 and are briefly described with the results obtained with them. Manuronate (10 mg./Kg., i.v.) was routinely used as the anticoagulant. Aqueous solutions of the compounds were prepared as concentrations such that the total volume of injection did not exceed 0.1 ml. for intra-arterial injection, and 1.0 ml. for intravenous injection.

Results.—The effects of mephentermine and its seven derivatives are summarized in Table I. Each compound was tested on at least three dogs and the comparative activity is expressed in relation to that of mephentermine with an activity ratio of 1.0. The relative potency of the derivative is expressed as either (*a*) the effect of an equivalent dose or (*b*) the dose that elicits an equivalent effect. Thus, the activity ratio of 0.5 signifies that an equivalent dose of the derivative produces half the effect of mephentermine, or twice the dose of the derivative produces an effect equivalent to mephentermine.

Pulmonary Vasodilatation.—The local action of a drug on the lung vessels was investigated by perfusing the left lower lobe at a constant flow with mixed venous blood from the dog's own right atrium (Fig. 5, top). Mephentermine (1 mg./Kg.) injected directly into the perfused pulmonary artery causes an immediate reduction in perfusion pressure. The 4-methoxy derivative

(compound 1364) induces a similar reduction in perfusion pressure when injected in a dose of 1 mg./Kg., indicating that this derivative causes pulmonary vasodilatation. Figure 6 depicts this response and includes the failure of 0.5 mg./Kg. of mephentermine to induce pulmonary vasodilatation. It is on this basis that the potency of the 4-methoxy derivative is expressed as 2.0.

All the other derivatives of mephentermine are weak pulmonary vasodilators (potency of 0.2) with one exception. The nitro derivative (compound 979) instead causes a rise in perfusion pressure, which means that this compound constricts the pulmonary vessels.

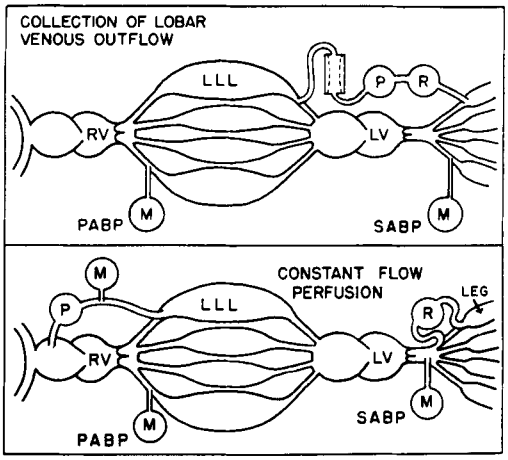


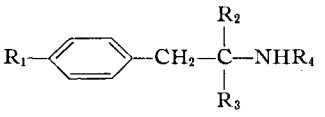
Fig. 5.—*Top.*—Collection of lobar venous outflow in a closed collapsible rubber reservoir, emptied by a Dale-Schuster pump (*P*), measured by a Shipley-Wilson rotameter (*R*), and returned to the animal's own femoral artery. Pulmonary arterial blood pressure (PABP) and aortic blood pressures (SABP) are measured by Statham transducers (*M*) recording on a Sanborn Poly Viso. *Bottom.*—Constant inflow perfusion of left lower lobe with mixed venous blood derived from the animal's own right atrium. Femoral arterial blood is shunted through a Shipley-Wilson rotameter (*R*). Perfusion pressure, arterial pressure of intact lobes, and aortic pressure are registered by Statham transducers (*M*).

Femoral Vasodilatation.—The same group of dogs that were subjected to lung perfusion were also utilized for the investigation of the femoral vascular bed by insertion of a rotameter for measuring arterial blood flow (Fig. 5). It can be noted in Fig. 6, *A* and *C*, that mephentermine and its 4-methoxy derivative, in a dose of 0.1 mg./Kg. injected directly into the femoral artery, cause an immediate increase in femoral blood flow. The responses as femoral vasodilatation induced by both compounds are equivalent in intensity (potency for both expressed as 1.0). The six other derivatives are uniformly able to dilate the femoral vascular bed, although most of them are weaker than mephentermine.

Pulmonary Venous Outflow.—Because of the various objections to the indirect methods of measuring cardiac output, it was decided to measure pulmonary venous outflow by a direct and unequivocal method (Fig. 5). A glass cannula was inserted into the vein of the left lower lobe. All the effluent blood was collected in a collapsible rubber reservoir at the same horizontal level as the cannula. The blood was instantaneously returned by means of a Dale-Schuster pump to the animal's own left atrium or femoral vein. The pump was set to empty the reservoir continuously so that pressure between the lobar vein and the reservoir was never allowed to increase. A Shipley-Wilson rotameter was inserted into the outflow side of the pump to obtain a graphic recording of the rate of blood flowing from the pulmonary vein. Although the pulmonary venous outflow measured by this method represents only one lobe, it is felt that it can be regarded as a reasonable criterion of flow in all lobes and of cardiac output.

The intravenous injection of mephentermine

TABLE I.—DERIVATIVES OF MEPHENTERMINE[a] AND THEIR PULMONARY VASCULAR EFFECTS BASED ON ACTIVITY OF MEPHENTERMINE EXPRESSED AS 1.0



Compound No.	R_1	R_2	R_3	R_4	Activity Ratios: Pulmonary Vaso-dilatation	Femoral Vaso-dilatation	Increased Pulmonary Blood Flow	Stimulation Myocardial Contraction
Mephentermine	...	CH_3	CH_3	CH_3	1.0	1.0	1.0	1.0
1364	4-CH_3O	CH_3	CH_3	CH_3	2.0	1.0	0.3	Variable
979	4-NO_2	CH_3	CH_3	CH_3	Constrict	1.0	Variable	Variable
1035	4-NH_2	CH_3	CH_3	CH_3	1.0	0.5	Variable	Variable
1420	...	CH_2OH	CH_2OH	CH_3	0.2	0.2	Variable	Variable
1239	...	CH_3	CH_3	CH_3—CH_2CH_2—CH_2	0.2	0.2	Variable	Variable
1240	...	CH_3	CH_3	$(CH_3)_2$—CH—CH_2	0.2	0.2	Variable	Variable
609	...	CH_3	CH_3	[b]	0.2	0.2	Variable	Variable

[a] Supplied by Dr. G. E. Farrar, Wyeth Laboratories, Radnor, Pa.
[b] Tertiary amine = CH_3 and $HOCH_2CH_2NHCH_2CO$.

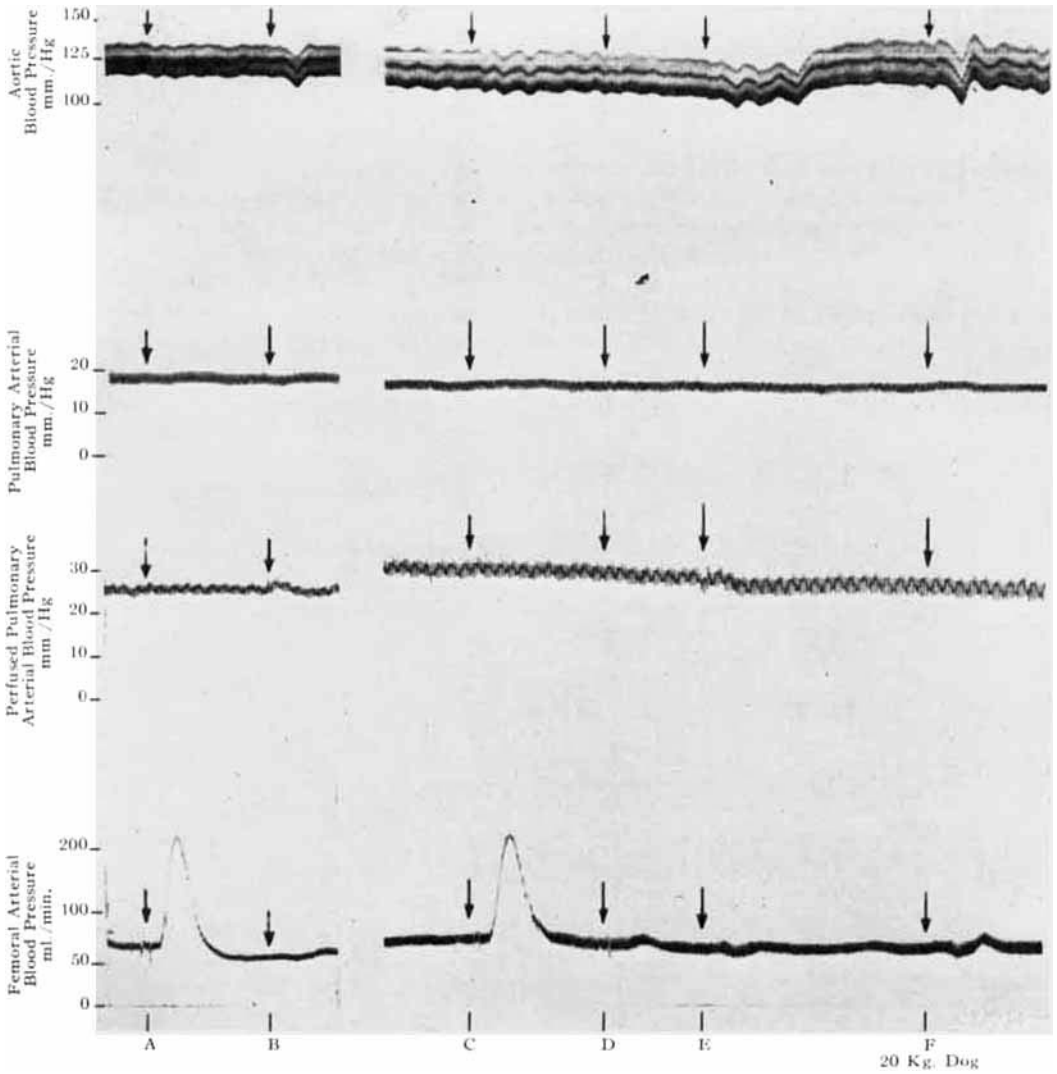


Fig. 6.—Comparative effects of mephentermine and 4-methoxy derivative. *A*, Mephentermine 0.1 mg./Kg. into femoral artery; *B*, mephentermine 0.5 mg./Kg. into perfused pulmonary artery (elapsed time between *B* and *C*, 20 min.); *C*, 4-methoxy derivative 0.1 mg./Kg. into femoral artery; *D*, saline 0.2 ml. into femoral artery; *E*, 4-methoxy derivative 0.5 mg./Kg. into perfused pulmonary artery; *F*, 4-methoxy derivative 1.0 mg./Kg. intravenously.

(1 mg./Kg.) causes an increase in pulmonary venous outflow (Figs. 7, *B*, and 8, C). The corresponding effect of the 4-methoxyderivative is a less intense increase in pulmonary venous outflow, with an activity ratio of 0.3 (Figs. 7, *A*, and 8, *A*). The other derivatives have variable effects on pulmonary venous outflow, either an increase or a decrease, and both may even appear in the same dog after repeated injections of the same compound. This variability is related to the biphasic action on the heart muscle which is described in the following paragraph.

Force of Myocardial Contraction.—The known stimulant action on the myocardium for mephentermine can be demonstrated by means of the Walton strain gauge. An intravenous injection (1 mg./Kg.) causes an increase in force of myocardial contraction, and this occurs simultaneously with the increase in pulmonary venous outflow (Figs. 7, *B*, and 8, *C*). An equivalent amount of the 4-methoxy derivative causes either an equally intense or less intense stimulation. After repeated injection of this compound, the stimulation may be converted to a temporary depression (Fig. 8, *D*). The variable effect on myocardial depression is related to the less intense increase in pulmonary venous outflow. The other derivatives have variable effects on myocardial force.

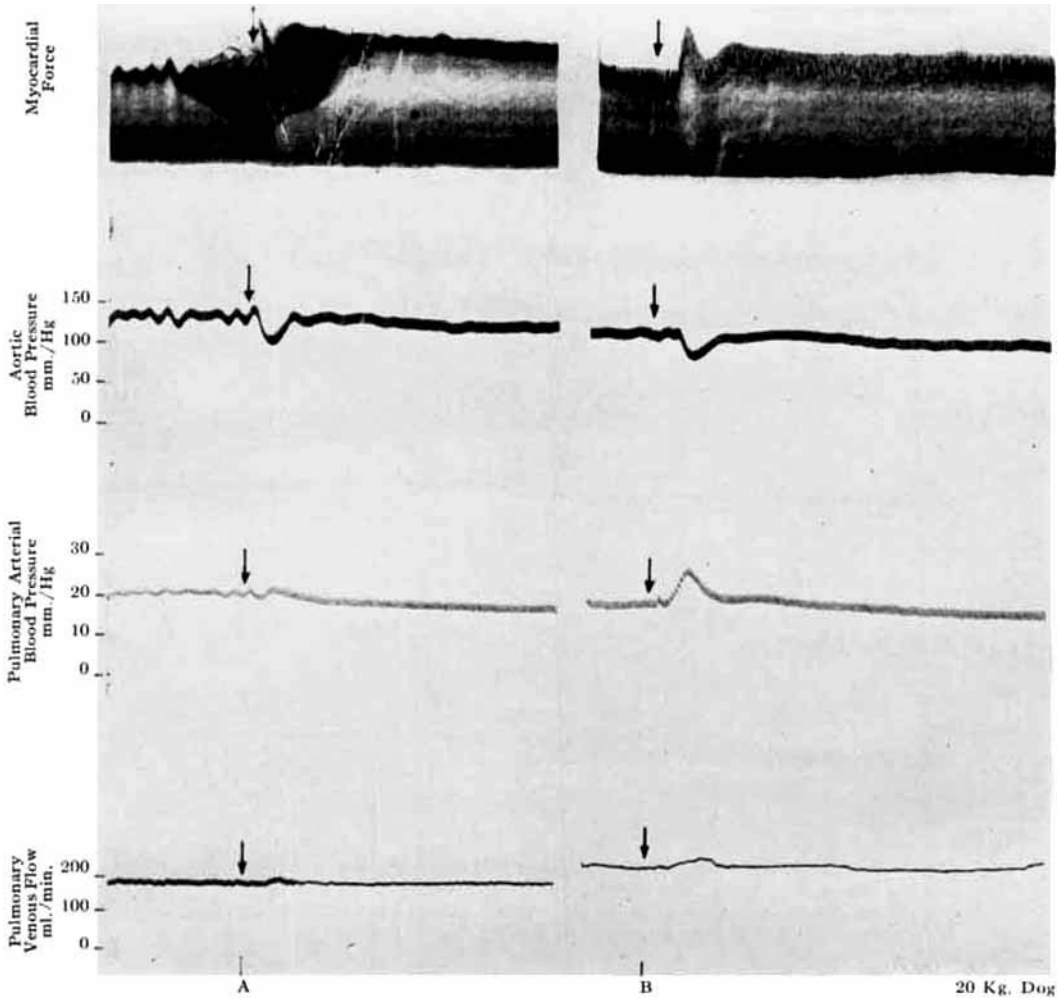


Fig. 7.—Comparative effects of mephentermine and 4-methoxy derivative on myocardial force, aortic blood pressure, pulmonary arterial blood pressure, and pulmonary venous outflow. *A*, 4-Methoxy derivative 1 mg./Kg. intravenously; *B*, mephentermine 1 mg./Kg. intravenously.

Pulmonary Blood Pressure.—The most usual response of pulmonary arterial blood pressure following the intravenous injection of 4-methoxy derivative is a fall. This fall most probably is related to the vasodilatation demonstrable in the perfused lung, which appears to be more important than the increase in pulmonary blood flow. In some instances the pulmonary arterial pressure is unchanged, and this can be explained by both increased flow and vasodilatation occurring to equalize each other. The pulmonary arterial pressure responses of mephentermine and the six derivatives (other than 4-methoxy) are variable, and are related to the effects on pulmonary blood flow and pulmonary vasomotor tone reported above.

Aortic Blood Pressure.—The aortic pressor response of mephentermine can be elicited by the intravenous injection of 1 mg./Kg. This has been explained by cardiac stimulation which has been documented by reports of various investigators (29–34). The pressor response is converted to a depressor response after the injection of the 4-methoxy derivative (Fig. 7) and this indicates that the systemic vasodilator action of mephentermine becomes of primary importance. The basic mechanism for this predominance induced by the 4-methoxy derivative is not known but may be an indication of cross-tachyphylaxis between the two compounds.

The behavior of aortic blood pressure following the intravenous injection of 4-methoxy derivative (1 mg./Kg.) is variable even in a dog receiving this compound for the first time. In a group of 6 dogs, a fall was encountered in 3, a rise in 1, and no change in 2. The fall can be explained by the

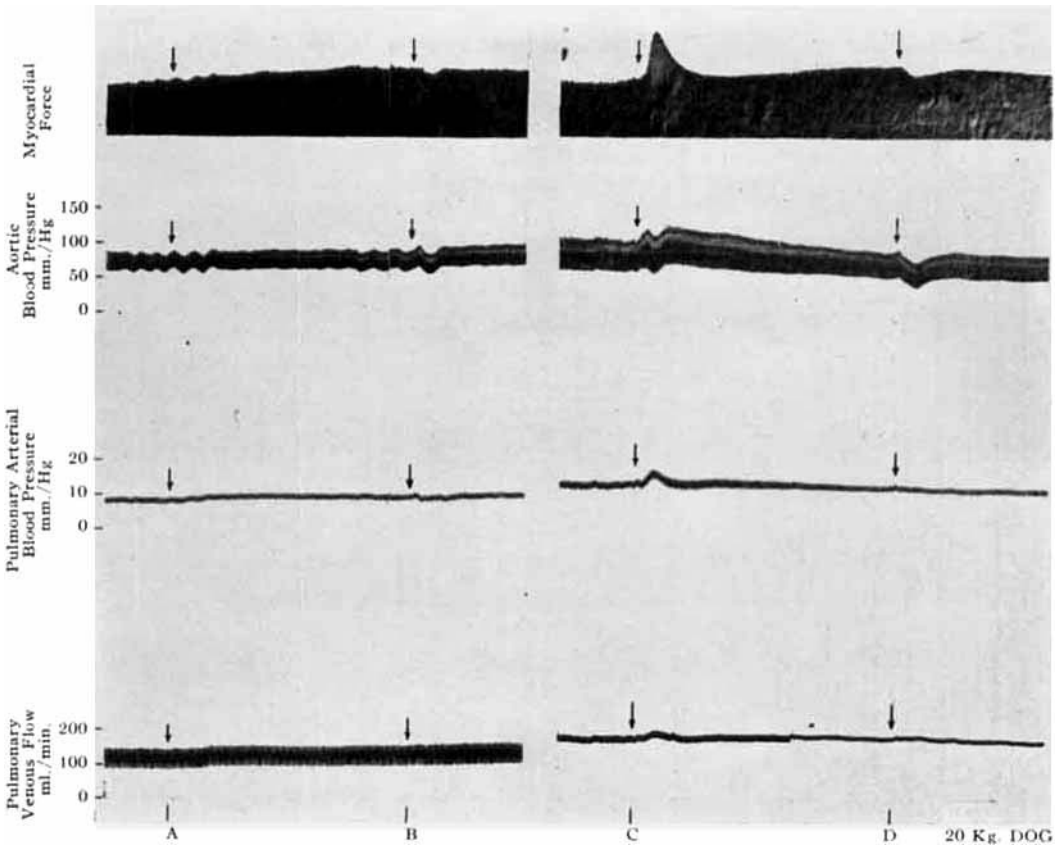


Fig. 8.—Comparative effects of mephentermine and 4-methoxy derivative on myocardial force, aortic blood pressure, pulmonary arterial blood pressure, and pulmonary venous outflow. *A*, 4-Methoxy derivative 1 mg./Kg. intravenously; *B*, 4-methoxy derivative 1 mg./Kg. intravenously; *C*, mephentermine 1 mg./Kg. intravenously; *D*, 4-methoxy derivative 2 mg./Kg. intravenously.

systemic vasodilatation, but final proof must await studies on systemic vascular beds other than the femoral.

Correlation of Chemical Structure to Pulmonary Vascular Action.—The effects of seven derivatives of mephentermine suggest the following generalizations. (*a*) The addition of a 4-methoxy radical in the phenyl ring to mephentermine causes an exaggeration of pulmonary vasodilator action but a reduction in the ability to increase pulmonary blood flow. The addition of a 4-amino radical does not reduce the pulmonary vasodilator action but reduces the systemic dilator action. (*b*) The substitution of a nitro radical converts the compound into a pulmonary vasodilator, the activity which is reduced by conversion of the methyl substituent in the terminal nitrogen to a longer aliphatic chain. A similar reduction in activity can be induced by substituting both methyl radicals in the α-carbon to two carbinol groups. All of these alterations reduce the vasodilator potency not only for the femoral vessels but also for the pulmonary vessels. (*c*) The ability to stimulate myocardial force is a feature common to all the derivatives tested, although this action is not a consistent one since depression may be encountered.

CONCLUDING REMARKS

The structure-activity relationships of antihistaminics, xanthines, and sympathomimetic amines have been reviewed. These drugs are able to (*a*) influence respiration by activation of pulmonary vagal receptors, (*b*) relax bronchiolar tone, (*c*) dilate pulmonary vessels, and (*d*) either decrease or increase pulmonary blood flow. The major shortcoming of active agents is their lack of selectivity for a specified structure in the lungs. They usually affect two or more reactive areas in the lungs and usually influence the heart and the systemic vessels.

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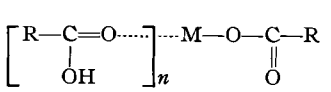
Research Articles

Complexation of Organic Acids and Bases with Their Salts in Aqueous Solution

By MICHAEL R. VALINOTI and SANFORD BOLTON

The solubilization of certain organic acids in aqueous solutions of their salts has been attributed to a complexation between the acid and anion species. In the present investigation solubility studies were used in an effort to ascertain the factors influencing such complexation and to determine the nature of the complexes formed. Benzoic acid, salicylic acid, *p*-hydroxy-, 2,4-dihydroxy-, 2,5-dihydroxy-, 2,6-dihydroxy-, and 3,4-dihydroxybenzoic acids, phenylacetic acid, adipic acid, barbituric acid, barbital, phenobarbital, and saccharin showed substantial increases in solubility in the presence of their sodium salts. Studies with atropine and procaine in solutions of their salts showed that similar complexes occurred in these systems. In some cases insoluble complexes were formed at high salt concentrations. Addition of hydroxyl groups to benzoic acid favors complexation. Quantitative evaluation of equilibrium constants could not be determined because of a concurrent salting out effect.

THE APPARENT increase in solubility of various organic acids in aqueous solutions of their alkali or alkaline earth metal salts has been attributed to the formation of an acid-anion complex (1–6). Ross and Morrison (1) have described solubility curves for various mandelic acid-metal mandelate systems and have noted the formation of complexes of acid-salt ratios of 1:1, 2:1, and 3:1. They proposed the following formula as the most favorable representation of the complex species



where n can be either 1, 2, or 3. Hoitsema (2) investigated the salicylic acid-sodium salicylate and hippuric acid-potassium hippurate systems

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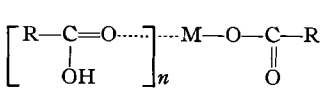
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and isolated, in each case, 1:1 "double salt" complexes. Marshall and Cameron (3) noted the appearance of, and characterized, an insoluble complex in the succinic acid-potassium succinate system. Kolthoff and Bosch (4), in studies designed to determine the activity coefficient of benzoic acid in solutions of various salts, observed an increase in the solubility of benzoic acid in the presence of up to 1 *M* sodium benzoate. Higuchi and co-workers (5) have noted an insoluble benzoic acid-ammonium benzoate complex; and Ongley (6) has listed, without interpretation, the increase in solubility of benzoic, salicylic, and 2,6-dihydroxybenzoic acids in 1 *M* solutions of their salts.

It was the purpose of the present investigation to attempt to elucidate the prerequisite factors and the nature of the binding forces involved in this type of complex. Solubility studies with a number of model compounds were used to determine the extent of complexation. Benzoic acid, phenylacetic acid, and a group of hydroxybenzoic acids were selected to determine the effect of some structural features of aromatic acids on the degree of complexation. Systems containing suitable monocarboxylic, straight chain acids proved to be too difficult to analyze because of the formation of colloidal soap solutions by their salts. Therefore, the adipic acid-sodium adipate system was investigated as an example of complex formation by nonaromatic carboxylic acids. Since no similar studies involving noncarboxylic acids have been reported in the literature, saccharin, barbituric acid, barbital, and phenobarbital complexes were also investigated. Likewise, because of the absence of published work of an analogous nature concerning bases and their salts, atropine and procaine complexes are reported here.

EXPERIMENTAL

Reagents.—All chemicals used were of the highest purity commercially available. The purity of the acids and bases was checked by (*a*) melting point, (*b*) solubility analysis, and (*c*) titration with standard acid or base. Those compounds failing to meet these criteria were recrystallized, usually from hot water. The melting points of the acids and bases used are listed in Table I. Solutions of salts that were not readily available were prepared either directly by adding an equivalent of sodium hydroxide to the acid or by preparing solutions from pure salt which was obtained by evaporation of the above solutions in a flash evaporator. With the exception of disodium adipate, monosodium salts were used as complexing agents for all acids. Atropine sulfate and procaine hydrochloride, both U.S.P. grade, were used as complexing agents for the bases.

Procedure.—A solubility method, similar to that employed by Higuchi and Lach (7), was used to follow the course of complexation. In all cases, a constant excess of the solid acid or base was added to varying concentrations of the salt solution. To determine the solubilizing effect of salt beyond its saturation point, calculated amounts of salt were added to the saturated solution. Because the small changes in volume resulting from the solubilization of the excess salt during complex formation could not be determined accurately, values plotted beyond the salt saturation point are, of necessity, approximations.

The mixtures, in duplicate, were placed in stoppered glass vials and rotated in a water bath at 30 ± 0.1° for at least 3 hours. In the base systems the shaking time was kept to a minimum to minimize hydrolytic decomposition.

In general, a 5-ml. sample of filtrate was withdrawn by means of a pipet fitted with a piece of rubber tubing plugged with cotton. The majority of the sodium salts were very soluble in water and, consequently, their solutions became very viscous as the saturation point was approached. This condition and the presence of large amounts of solid acid, complex, or salt in the system made withdrawal of accurate aliquots unfeasible in some instances. As a consequence, the solubility analyses were carried

TABLE I.—SOLUBILITY CHARACTERISTICS AND INTERACTIONS OF SOME ORGANIC ACIDS AND BASES WITH THEIR SALTS

Compound	M.P., °C.	Solubility Compound, Mole/L. × 10^2	Solubility Salt, Mole/L.	Apparent K_1, L./Mole	Insoluble[a] Complex
Benzoic acid	122	3.07	2.98	0.5	none
Salicylic acid	158	1.88	4.3	0.9	1:1
p-Hydroxybenzoic acid	215–216	5.71	2.6	0.7	1:1, 1:2
2,4-Dihydroxybenzoic acid	225 decompn.	4.87	0.60	2.2	none
3,4-Dihydroxybenzoic acid	201–202 decompn.	11.4	0.85	0.9	1:1
2,5-Dihydroxybenzoic acid	204–206 decompn.	17.4	1.45	2.8	1:1
2,6-Dihydroxybenzoic acid	164–167 decompn.	7.54	1.60	1.6	none
Phenylacetic acid	76–77	14.7	3.00	1.2	none
Adipic acid	152–154	19.6	2.00	7.5[b]	2:1
Saccharin	224–226	1.95	3.00	1.9	none
Barbituric acid	247–250 decompn.	11.1	0.10	0.9	1:1
Barbital	188–191	4.38	0.90	0.5	none
Phenobarbital	172–176	0.612	2.65	1.0	1:4
Atropine	116–117	1.00	1.03	13.	none
Procaine	59–61	1.92	2.80	4.8	none

[a] Ratios given are for acid:salt. [b] Calculated on basis of 2:1 complex.

out to a point of salt concentration beyond which the results were believed to be inaccurate.

The clear aliquot was titrated potentiometrically with standard sodium hydroxide or hydrochloric acid, unless the titration was readily amenable to the use of phenolphthalein. Barbital and phenobarbital, which were titrated potentiometrically, required the prior addition of ethanol to the aqueous solutions in order to yield a clearly discernible end point. For a number of compounds, in concentrated salt solutions, the end point could not be clearly detected. In these cases, to eliminate most of the salt, the aqueous aliquot was first extracted with ether (chloroform was used in the case of atropine), the organic solvent evaporated, and the residual acid or base titrated as before. Preliminary determination of known concentrations of the acids and bases in concentrated solutions of their salts substantiated the accuracy of the above method.

The acid:salt ratios of the insoluble complexes were determined by analyzing the precipitates in the appropriate containers for acid content.

RESULTS

Benzoic and Monohydroxybenzoic Acids.—Figure 1 illustrates the results obtained for benzoic, salicylic, and *p*-hydroxybenzoic acids. The benzoic acid curve, up to 1 *M* sodium benzoate, parallels the previously reported data of Kolthoff and Bosch (4) whose study was made at 25°. No insoluble complex is formed in this system; the plateau region at high salt concentration indicates that the solution, in this range, is saturated with respect to salt. Salicylic acid shows a significantly greater complexing tendency than benzoic acid. The decrease in acid solubility, observed at low salt concentrations, is probably a salting out effect, a result also observed by Hoitsema (2). The termination of the sharply ascending curve is caused by the appearance of an insoluble complex containing acid and salt in a 1:1 ratio. Further addition of salt results in depletion of acid in solution as more complex is precipitated (7). *p*-Hydroxybenzoic acid, like benzoic acid, shows no initial decrease in solubility. A 1:1 complex precipitated along the first plateau region on this curve. The shape of the terminal portion of the curve and the analysis of the solid phase in that region strongly suggest the formation of a new insoluble species containing acid and salt in a ratio of 1:2.

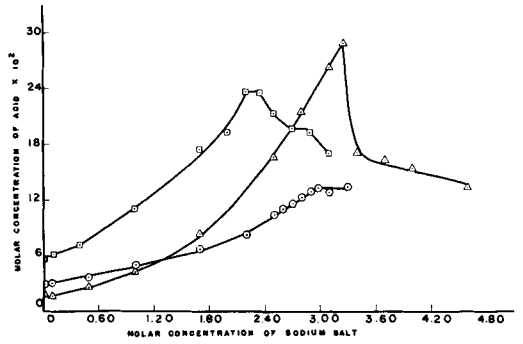


Fig. 1.—Apparent solubilization of benzoic ⊙, salicylic △, and *p*-hydroxybenzoic ▣ acids by their sodium salts in water at 30°.

Dihydroxybenzoic Acids.—Figures 2 and 3 show the results of the dihydroxybenzoic acid interactions studied. Among these acids, only the 2,6-dihydroxy derivative shows an initial decrease in solubility. This acid and 2,4-dihydroxybenzoic acid form soluble complexes only. Analysis of these curves according to a method suggested by Higuchi and Lach (7) indicated formation of 1:1 complexes. In both the 3,4-dihydroxy and 2,5-dihydroxybenzoic acids, precipitation of a 1:1 complex occurs after an initial increase in the apparent solubility of the acid.

Phenylacetic and Adipic Acids.—Figure 4 shows the interaction of phenylacetic and adipic acids with their respective sodium salts. In the phenylacetic acid system, a salting out effect is apparent at low salt concentrations, as evidenced by the appearance in the solubility curve of an initial flattened portion followed by an abrupt rise. Adipic acid forms an insoluble complex with its sodium salt. Analysis of the precipitate on the descending portion of the curve was indicative of an insoluble species containing acid to salt in a 2:1 ratio. At the terminal segment of the curve, the solution is saturated with salt and the solid phase consists of insoluble complex plus salt.

Noncarboxylic Acids.—Figure 5 shows the results obtained for saccharin. A pronounced initial decrease in acid solubility is noted, followed by solubilization up to the salt saturation point. Further addition of salt along the plateau region failed to precipitate a complex species.

Figure 6 illustrates the varied behavior of barbituric acid and its derivatives. The barbituric acid-barbiturate complex is the least soluble of all

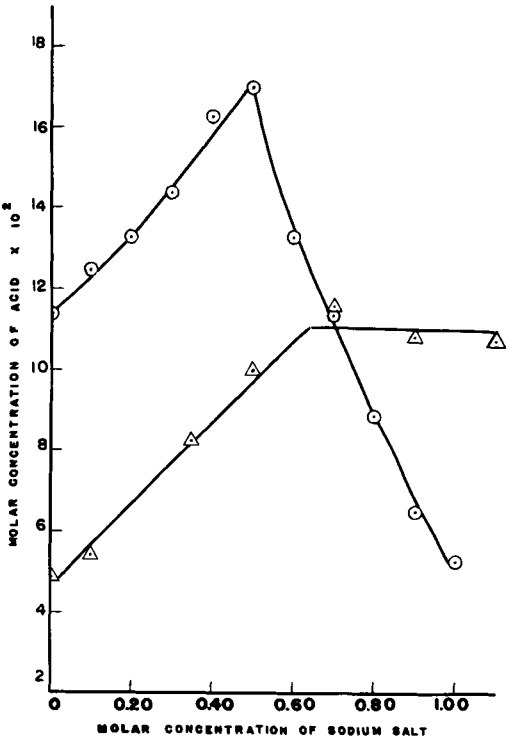


Fig. 2.—Apparent solubilization of 2,4-dihydroxybenzoic △ and 3,4-dihydroxybenzoic ⊙ acids by their sodium salts in water at 30°.

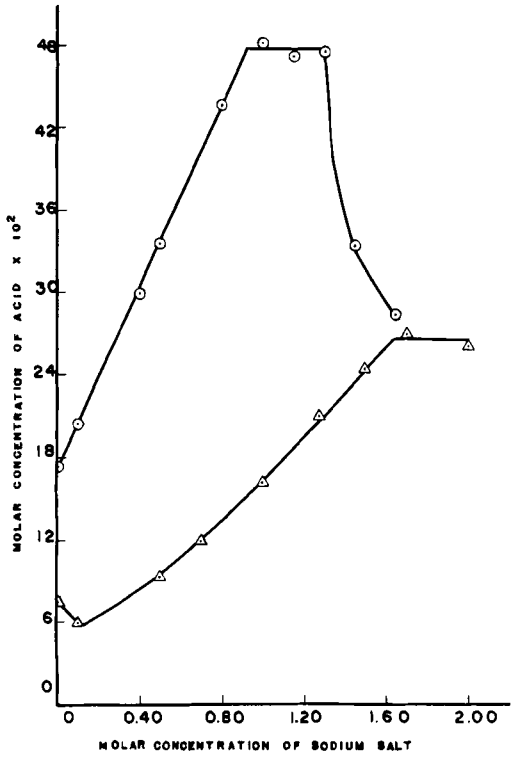


Fig. 3.—Apparent solubilization of 2,5-dihydroxybenzoic ⊙ and 2,6-dihydroxybenzoic △ acids by their sodium salts in water at 30°.

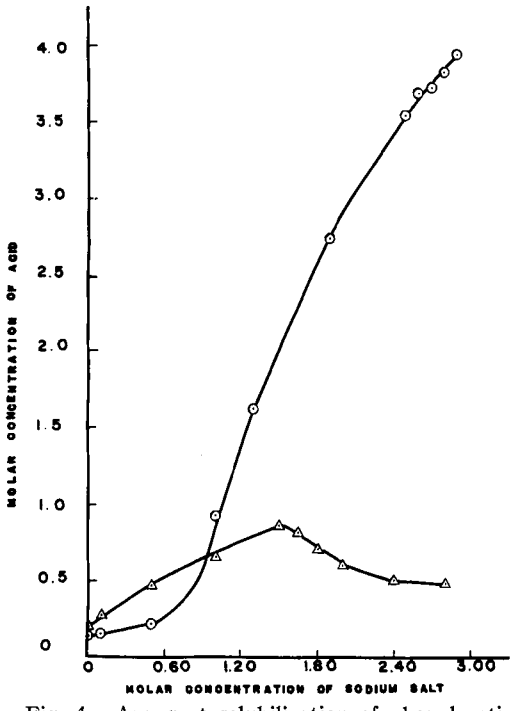


Fig. 4.—Apparent solubilization of phenylacetic ⊙ and adipic △ acids by their sodium salts in water at 30°.

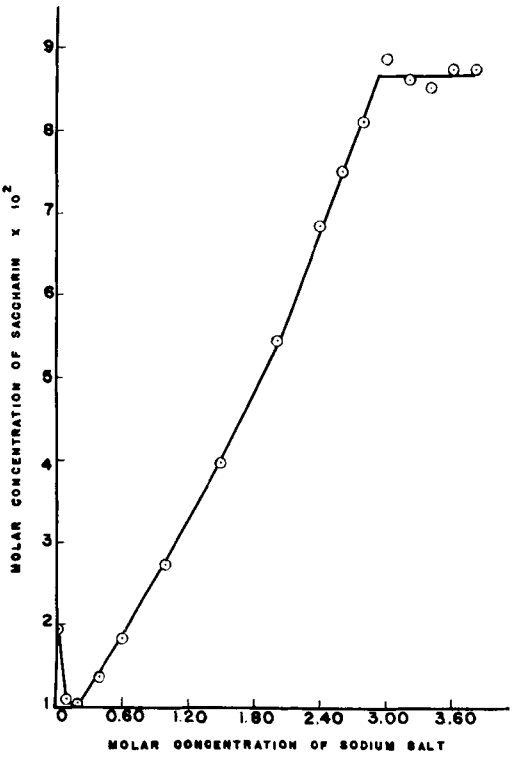


Fig. 5.—Apparent solubilization of saccharin by sodium saccharin in water at 30°.

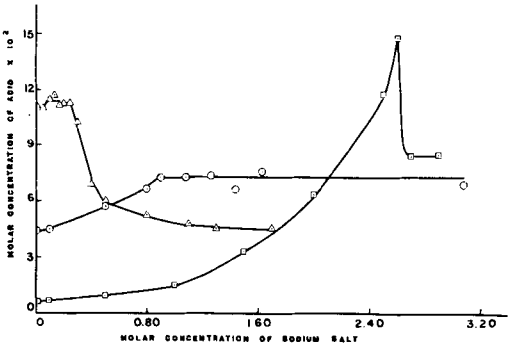


Fig. 6.—Apparent solubilization of barbituric acid △, barbital ⊙, and phenobarbital ◻ by their sodium salts in water at 30°.

complexes noted in this study. After an initial decrease, a small increase in acid solubility is observed, followed by the appearance of a 1:1 complex. Again, along the terminal portion of the curve, the system is saturated with salt and is invariant. Barbital is solubilized up to the salt saturation point, and no insoluble complex is formed. In the case of phenobarbital, the relatively small increase in solubility at low salt concentrations may indicate a salting out effect, somewhat masked by complex formation (similar to the phenylacetic acid system). With increasing salt concentration, however, solubilization increases rapidly. The insoluble complex which appeared at high salt concentration corresponded closely to an acid:salt ratio of 1:4.

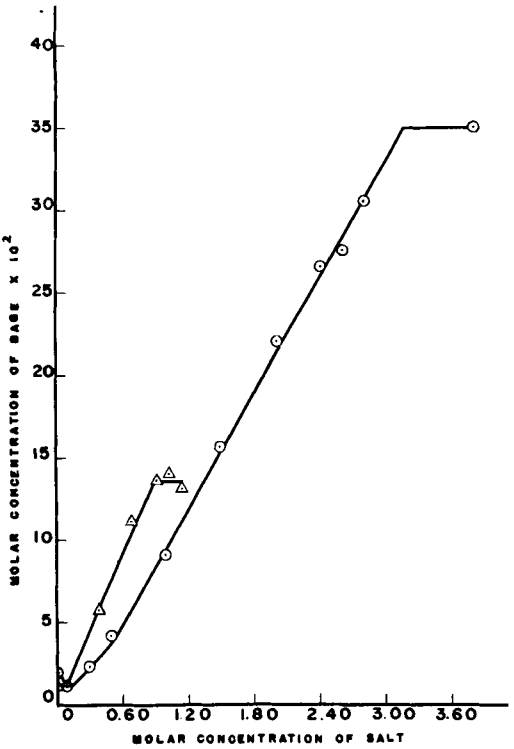


Fig. 7.—Apparent solubilization of atropine by atropine sulfate △ and procaine by procaine hydrochloride ⊙ in water at 30°.

Bases.—The complexation of atropine and procaine with their salts is shown in Fig. 7. The curves are similar in nature to those observed in the acid systems. In both of these cases no insoluble complex appears. In the procaine system, the observed increase in solubility of both the base and salt species due to complex formation indicated formation of a 1:1 complex (7).

DISCUSSION

The fact that complex formation in these systems is accompanied by an inseparable salting out effect renders accurate evaluation of stability constants impossible. Nevertheless, constants were approximated for 1:1 complexes, neglecting salting out effects. These constants were calculated, according to a previously reported method (7), from the initial portions of the curves where 1:1 complexation could be considered to be the predominant equilibrium. Although this approach, admittedly, yields only rough approximations, the results appeared to give a reasonable indication of the extent of complex formation (Table I). In any case the constants, thus calculated, give low values since salting out effects tend to obscure complexation effects.

The K_1 value for barbituric acid was approximated from the final invariant portion of the curve. In this region, the solution is saturated with both complex and salt. The solubility of the complex may be estimated from the initial rise in the solubility curve, and the solubility of the salt is known. The concentration of free acid in these solutions may be calculated as follows

(free acid) = (total acid in solution) − (complex)

and

$$K_1 = \frac{(\text{complex})}{(\text{free acid})\,(\text{free salt})}$$

Since physical properties, such as melting point and solubility, are measures of intermolecular forces and may give insights into complexation forces, these data are included in Table I.

In the series of benzoic acid and its hydroxy derivatives, there appears to be little or no correlation of complexing activity with the indicated physical properties. This fact suggests that the interaction is the result of an interplay of many factors. The solubility of the complexes formed also shows no obvious relation to any single property of these acids. The fact that the addition of hydroxy groups to the benzoic acid nucleus results in an increase in the degree of complexation suggests that binding may occur through hydrogen bonds as well as through the metal atom as previously suggested (1). It is of interest to note that the 2-substituted hydroxy acids showed the strongest complexation in all cases.

The limited, comparable data reported by Ross and Morrison (1) for mandelic acid indicates that at low salt concentrations this acid complexes more strongly with its sodium salt than does phenylacetic acid with its salt. The introduction of a hydroxyl group in mandelic acid may again be offered as an explanation of this difference.

Significant complexation occurred in the nonaromatic acid and the base systems; but the degree of binding, as well as the solubility of the complexes, again appeared to be nonselective.

The variety of complex systems tested indicates that interaction between organic acids or bases and their salts is a widespread phenomenon. There does not appear to be any overall structural requirements although, in a given class of compounds, certain structural variations are probably influential. Despite a wide range of melting points and solubilities of the acids, bases, and their salts, these factors alone do not seem to provide a basis for correlation with complexing activity.

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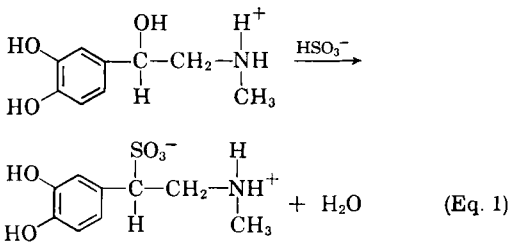
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Stabilization of Epinephrine against Sulfite Attack

By SIDNEY RIEGELMAN and ELIEZER Z. FISCHER†

Sulfite and bisulfite ions are known to attack the optically active side chain of epinephrine in the absence of oxygen by an S_N2 mechanism. Evidence is presented whereby chelation of the catechol nucleus with boric acid results in marked stabilization of epinephrine from this type of degradation. The effects of variation of the boric acid and hydrogen ion concentration are investigated. The equilibrium reaction of boric acid and epinephrine are reported in detail and the stability constant for the chelate is evaluated.

In 1958 Schroeter and Higuchi (1, 2, 3) reported on a reaction of epinephrine with bisulfite in an oxygen-free atmosphere. The mechanism of this reaction appears to involve nucleophilic attack of sulfite at the partially positive carbon of the epinephrine side chain to yield a carbon-sulfur bond. There is a loss of optical activity simultaneous to epinephrine and bisulfite loss; the result is a zwitterionic sulfonate



(Eq. 1)

The latter compound had been prepared by Tomino (4) and by Schroeter (5), although the former worker proposes that the sulfonate group is attached at the β-carbon atom.

Studies of the kinetics of the reaction by Higuchi and Schroeter definitely confirm the reaction to be a second-order reaction, dependent on both the concentration of the epinephrine and of the bisulfite. Epinephrine analogs and model compounds were examined by these workers (2) who pointed to the general nature of this type of nucleophilic attack on *p*-substituted benzyl alcohol derivatives. *Meta*-substituted derivatives did not react. The results led to the suggestion that the *p*-quinoid structure of the benzyl alcohol derivatives is the reactive form.

Based on this background information, the present authors postulated that chelate formation between boric acid and the catechol grouping of epinephrine might affect the overall rate of sulfite attack on the compound. Chelate formation of boric acid with polyhydroxy compounds is well known (6, 7). Boric acid chelates of catechol derivatives were first studied by Boeseken and Mijs (8) whose main interest was directed toward preparation of the optically active forms of the chelates. Recently Näsänen (9) has studied the chelate formation of catechol-3,5-disulfonic acid with boric acid by a potentiometric method. The titration of dilute mixed solutions of boric and catechol disulfonic acids with sodium hydroxide solution revealed the formation of only a one-to-one chelate. Since boric acid was found to stabilize epinephrine against sulfite attack, it was deemed important to determine the stability constant for the formation of boro-epinephrine chelate in order to elucidate the quantitative aspects of the stabilization. The results of the degradation and chelate formation studies are the basis of this report.

EXPERIMENTAL

Kinetic Studies.—Epinephrine solutions were prepared at varying concentrations in phosphate or boric acid buffers at varying pH. The pH was determined at 25°. These solutions were filled into hard-glass ampuls, flushed with nitrogen, degassed in a vacuum and once again filled with nitrogen, and then sealed. Identical solutions were prepared containing sodium bisulfite. As mentioned earlier, the reaction of epinephrine with bisulfite had been shown to be a bimolecular reaction with simultaneous loss of optical activity and sulfite. In order to accelerate the rate of reaction, a swamping excess of sodium bisulfite was used in the reaction system converting the system to a pseudo first-order reaction. Thus it was possible to make the observations on samples equilibrated in a constant temperature bath at 25°. Individual ampuls were removed periodically and the optical activity of the solution at 25° was determined on a Rudolph research polarimeter using filtered sodium light (589 mμ) and a 2.0-dm. microtube. The chemical activity of the epinephrine was verified in a number of the solutions by isolation of the triacetyl derivative according to the method reported by Higuchi, Sokoloski, and Schroeter (10). The bisulfite concentration of the ampuls was also determined by iodometric titration.

Chelate Formation Studies.—Solutions of epinephrine, phenylephrine, and boric acid were prepared

Received April 28, 1961, from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Accepted for publication June 21, 1961.
This study was supported in part by a grant-in-aid from Barnes-Hind Laboratories, Sunnyvale, Calif.
Presented to the Scientific Section, A. Ph. A., Chicago meeting, April 1961.
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using exact equivalents of hydrochloric acid to prepare the salts from the free bases. Sodium chloride was added to adjust the ionic strength of the solutions. The solutions were transferred to a four-necked flask and degassed with nitrogen purified with a vanadous sulfate solution to remove residual traces of oxygen (11). The potentiometric measurements were made using the Beckman standard combination electrode (No. 39142) and the Beckman model G pH meter. Standard sodium hydroxide solution was delivered from a 5-ml. microburet.

Chelate formation was studied spectrophotometrically using 0.4% epinephrine solutions in the presence of an excess of boric acid. The solutions were adjusted to varying pH's by the addition of 10 *N* sodium hydroxide. The pH's of the solutions were measured as noted above. The ultraviolet absorption spectrum was recorded using a Cary model 11 spectrophotometer.

RESULTS AND DISCUSSION

Typical degradative attack of sulfite on epinephrine in the absence of boric acid is shown in Fig. 1. In these solutions, the concentration of epinephrine was held at 0.082 *M* and the concentration of sodium bisulfite was 0.96 *M*. It is evident from Fig. 1 that the reaction follows first-order kinetics under these conditions. The amount of sulfite consumed was completely accounted for by the simultaneous loss in epinephrine, as measured chemically, or by the change in specific rotation of the solutions. It is noted that in the absence of a complexing agent the rate of sulfite attack increases with decrease in hydrogen-ion concentration. This is in agreement with the observations of Higuchi and Schroeter (2) at elevated temperatures and different concentration conditions.

When identical solutions in respect to epinephrine and sulfite concentrations are prepared containing 0.318 *M* boric acid adjusted to the same pH, an interesting effect takes place relative to the rate of sulfite attack. Figure 2 presents the data for the deterioration of epinephrine at the same pH values as shown in Fig. 1. In the presence of boric acid, the rate of sulfite attack is reduced as the hydrogen ion concentration decreases. The half-life for epinephrine under the reaction conditions at pH 6.0 in the absence of boric acid was 195 hours, while in the presence of boric acid the half-life was found to be 267 hours. At pH 7.5, however, the half-life of epinephrine was found to be 74 hours in the absence, and 1,270 hours in the presence of boric acid. It might be postulated that epinephrine is increasingly chelated by the boric acid molecules as the pH is made more alkaline, and that the chelated epinephrine is far less susceptible to sulfite attack than free epinephrine. This is analogous to the findings of Higuchi and Lachman in their studies on the effect of complexation of benzocaine with caffeine (12). In that latter study it was concluded that the complexed form of the drug does not undergo any perceptible cleavage at the ester linkage of the benzocaine. In order to ascertain more facts relative to the stability of the boro-epinephrine chelate to sulfite attack, it was necessary to determine the stability constant for the reaction.

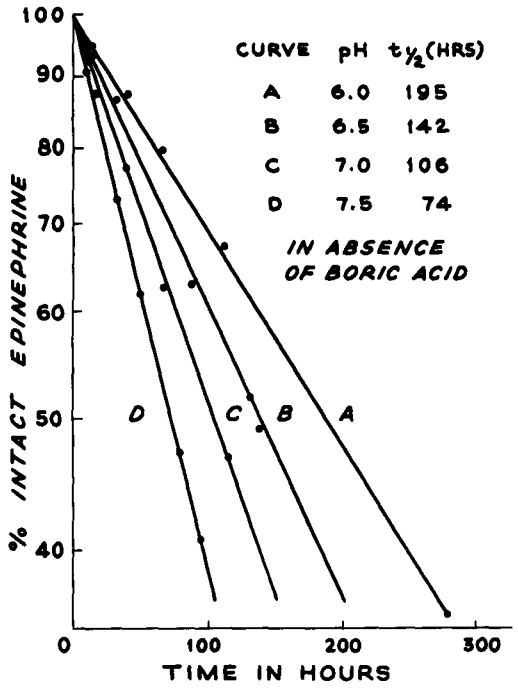


Fig. 1.—The per cent epinephrine remaining in a 1.5% (0.082 *M*) solution containing 10% (0.96 *M*) sodium bisulfite. Solutions were stored at 25° in sealed ampuls in a nitrogen atmosphere in the absence of oxygen. The solutions contained no boric acid.

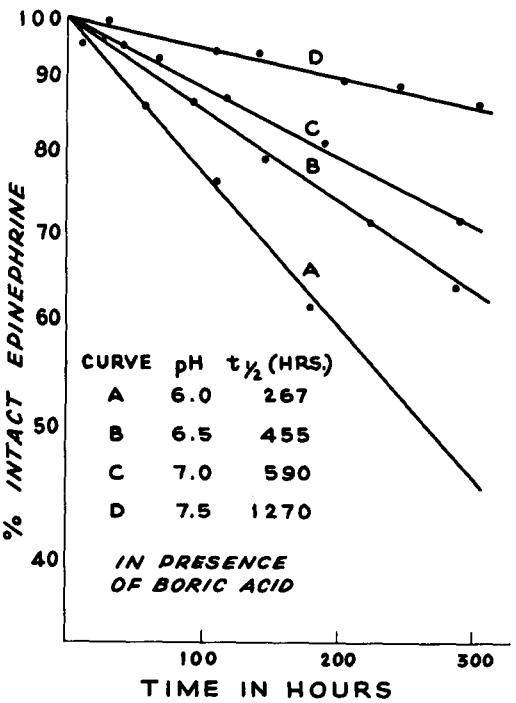


Fig. 2.—The per cent epinephrine remaining in a 1.5% (0.082 *M*) solution containing 10% (0.96 *M*) sodium bisulfite and 2.0% (0.318 *M*) boric acid. The solutions were stored at 25° in sealed ampuls in a nitrogen atmosphere in the absence of oxygen.

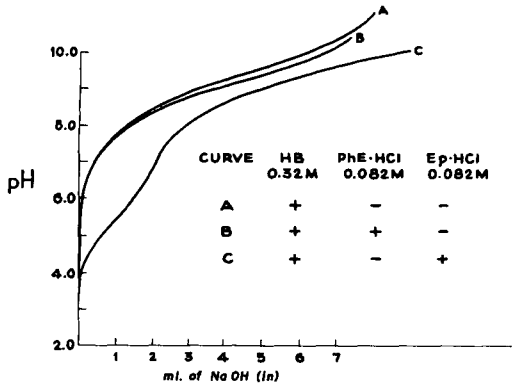


Fig. 3.—Titration of 0.318 *M* boric acid alone (*A*), in the presence of 0.082 *M* phenylephrine hydrochloride (*B*), and in the presence of 0.082 *M* epinephrine hydrochloride (*C*).

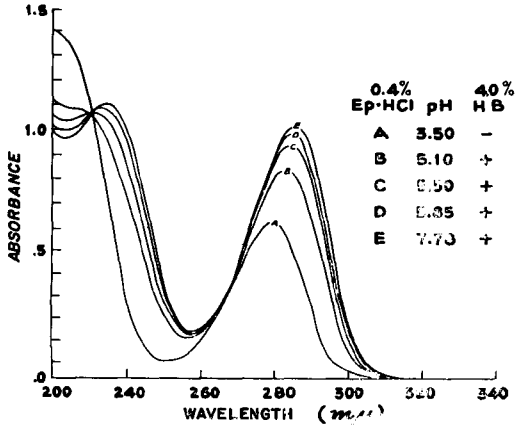
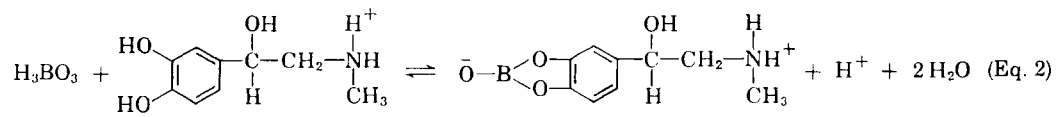


Fig. 4.—Ultraviolet absorption spectra of 0.022 *M* epinephrine alone and in the presence of 4% (0.65 *M*) boric acid at various hydrogen ion concentrations, path length 0.1 mm.

Figure 3 presents the titration curves of 0.32 *M* boric acid alone (*A*), in the presence of 0.082 *M* phenylephrine hydrochloride (*B*), and in the presence of 0.082 *M* epinephrine hydrochloride (*C*). Phenylephrine, of course, differs from epinephrine in that it is a *m*-cresol amine while epinephrine is a catechol amine derivative. Thus epinephrine is able to form a chelate through its dihydroxy structure and the absence of any chelate formation with phenylephrine indicates that the site of binding does not involve the side chain. Curve *B* is slightly shifted relative to the pure boric acid titration curve (*A*) due to the simultaneous neutralization of the first acid function of phenylephrine hydrochloride. Similar titration data for the 1:1 ratio of boric acid to epinephrine have been reported by Riegelman, *et al.* (13). These data may be represented by the reaction



(Eq. 2)

The equilibrium constant for the reaction represented by Eq. 2 may be represented as follows

$$K = \frac{[H^+][EpB^-]}{[Ep][Hb]} \qquad \text{(Eq. 3)}$$

where [HB], [Ep], and [EpB^-] represent the equilibrium concentrations of the boric acid, epinephrine, and boro-epinephrine chelate, respectively. Activity coefficients are ignored. In analogy to the results of Näsänen (9) who found only the 1:1 chelate, one might expect only a 1:1 chelate under the present conditions where the concentration of boric acid is equal to or exceeds the concentration of the epinephrine. This is also verified by the reaction of only one mole of base per mole of epinephrine added and the existence of an isosbestic in the absorption spectral curves (Fig. 4).

The stability constant for the reaction can best be calculated in the pH region where base is not consumed by the excess of boric acid present. Under these conditions the amount of base consumed is equal to the amount of chelate formed, and each of the equilibrium concentrations represented in Eq. 3 are thereby easily calculated from simple stoichiometric relationships. The data in Table I list the results from the calculations at two different ratios of boric acid to epinephrine. Since the ionic strength of the two systems differ, the difference in the calculated stability constants may be due to ionic strength effect or to other experimental considerations. Näsänen found a pK of 4.0 for catechol disulfonic acid with relatively large ionic strength effects.

Figure 4 presents the ultraviolet absorption spectra of epinephrine 0.022 *M* in the presence of 0.65 *M* boric acid at varying hydrogen ion concentrations. Since the extinction coefficient of boro-epinephrine chelate is not known with certainty, a modification of the method of Rosenblatt (14) must be used to calculate the stability constant from the absorption data. Equation 3 may be rewritten as

$$K' = [HB]K = \frac{[H^+][EpB^-]}{[Ep]} \qquad \text{(Eq. 4)}$$

since under the condition of the spectrophotometric analysis, [HB] $\sim$ constant. Substituting the appropriate extinction coefficients, we have

$$\frac{1}{K'} = \frac{1}{[HB]K} = \frac{\epsilon_{(EpB^-)} - \epsilon_n}{\epsilon_n - \epsilon_{(Ep)}} \frac{1}{[H^+]_n} \qquad \text{(Eq. 5)}$$

where ϵ_{Ep} = specific extinction coefficient of Ep; $\epsilon_{(EpB^-)}$ = specific extinction coefficient of EpB^-, and ϵ_n = specific extinction coefficient of a mixture of each component at the hydrogen ion concentration, $[H^+]_n$, or

$$-[H^+]_n\,\epsilon_n\left(\frac{1}{K'}\right) + [H^+]_n\left(\frac{\epsilon_{Ep}}{K'}\right) + \epsilon_{(EpB^-)} = \epsilon_n \qquad \text{(Eq. 6)}$$

This is a linear equation of four terms and three

unknowns since $[H^+]_n$ and ϵ_n are measurable quantities. This may be solved by use of a third-order determinant, or

$$[HB]K = K' = \frac{\begin{vmatrix} [H^+]_1\ \epsilon_1 & [H^+]_1\ 1 \\ [H^+]_2\ \epsilon_2 & [H^+]_2\ 1 \\ [H^+]_3\ \epsilon_3 & [H^+]_3\ 1 \end{vmatrix}}{\begin{vmatrix} \epsilon_1 & [H^+]_1\ 1 \\ \epsilon_2 & [H^+]_2\ 1 \\ \epsilon_3 & [H^+]_3\ 1 \end{vmatrix}}$$

The results from the analysis at two wavelengths are given in Table II. The calculated stability constants from the spectrophotometric studies compare surprisingly well with those determined by the titration method.

TABLE I.—CALCULATION OF THE STABILITY CONSTANT FOR THE BORO-EPINEPHRINE CHELATE FROM THE TITRATION DATA

Composition	pH	pK
Ep = 0.082*M* HB = 0.082*M*	4.66	4.65
	4.78	4.63
	5.07	4.66
	5.18	4.65
	5.45	4.69
	5.72	4.69
		4.66 ± 0.04[a]
Ep = 0.082*M* HB = 0.32*M*	3.88	4.76
	4.30	4.71
	4.68	4.70
	4.88	4.71
	5.00	4.72
		4.71 ± 0.01[a]

[a] Mean ±95% confidence interval.

TABLE II.—CALCULATION OF STABILITY CONSTANT FOR THE BORO-EPINEPHRINE CHELATE FROM U. V. SPECTROPHOTOMETRIC DATA

Composition	pH	A_{285} mμ	A_{240} mμ
Ep = 0.022 *M* HB = 0.648 *M*	5.10	0.835	0.725
	5.50	0.932	0.880
	5.83	0.950	0.960
	$pK_{285\ m\mu}$ = 4.66		
	$pK_{240\ m\mu}$ = 4.71		
[HB] equil. was assumed to be 0.625 *M*			

Closer examination of the sulfite attack on epinephrine in the presence of boric acid may now be made. The stability constant calculated above indicates that the reaction illustrated by Eq. 2 is predominantly shifted to the left at low pH. The ratio of the boric acid to the epinephrine and the hydrogen ion concentration both affect the concentration of free epinephrine in the system. Since there is a practical limit to the concentration to which we can increase the concentration of boric acid, the most important variable in the reaction is the hydrogen ion concentration. As the hydrogen ion concentration is decreased, the boro-epinephrine chelate concentration is increased, and the preparation is thereby stabilized to sulfite attack.

At pH's above 6.0 and at relatively high concentrations of boric acid, it becomes increasingly difficult to estimate the concentration of free epinephrine directly from Eq. 3. This is due to the fact that boric acid forms autocomplexes in the concentration ranges utilized which, in turn, affect the apparent dissociation constant of the boric acid (15, 16). In the pH range where the sulfite attack was studied, a portion of the base was utilized to titrate boric acid and polyboric acids.

The amount of free and chelated epinephrine can be evaluated by an alternative approach. Using the same ratio of boric acid to epinephrine as utilized in the deterioration studies (3.9/1), a series of solutions were made at 0.022 *M* epinephrine and 0.084 *M* boric acid and adjusted to the exact pH values used in the deterioration studies. The ultraviolet absorption spectra of the solutions were examined in a 0.1-mm. path length. Since two isosbestics were observed, solutions contained only two absorbing species. The spectrum of a solution of epinephrine hydrochloride in the absence of boric acid and a mixture of boric acid and epinephrine at pH 9.0 was also recorded. By analysis of the system at two wavelengths, the concentration of free epinephrine was determined for each solution. The data are recorded in Table III. Column 2 lists the fraction of free epinephrine as determined spectrophotometrically. In analogy to the approach of Higuchi and Lachman, the overall rate of reaction of epinephrine with sulfite is dependent on the reaction rate for the free and chelated epinephrine, or

$$k_{obs} = k_f F_f + k_c F_c \qquad \text{(Eq. 8)}$$

where the subscripts f and c stand for the free and chelated epinephrine, respectively, and F = the fraction of each form. If we assume that the rate of degradation of the chelated epinephrine is extremely small compared with the free, and substituting reciprocal half-lives for the respective rate constants we have, $F_f = (t_{1/2})_f/(t_{1/2})_{obs}$.

TABLE III.—COMPARISON OF CALCULATED AND EXPERIMENTAL ESTIMATION OF FREE EPINEPHRINE CONCENTRATION

pH	Calculated Free Epinephrine	Observed Free Epinephrine
6.0	0.45	0.45
6.5	0.22	0.315
7.0	0.069	0.15
7.5	0.031	0.058

Column 3, Table III lists the calculated fraction free from the data given in Figs. 1 and 2. It should be recalled that the solutions used in the degradation studies included 0.96 *M* sodium bisulfite, which produced an ionic strength far different from that used in the spectrophotometric evaluations. In addition, the latter measurements utilized solutions of identical ratio but diluted 3.75-fold in order to measure their absorbancies. While the deviation in columns 2 and 3 may be due to the changes in experimental conditions, an assumption that $k_c \leq 0.05\ k_f$ can approximate the values observed.

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Effect of Boric Acid and Bisulfite on the Rate of Oxidation of Epinephrine

By SIDNEY RIEGELMAN and ELIEZER Z. FISCHER†

An investigation of epinephrine and sulfite oxidation in the presence and absence of light was undertaken to determine what effect complexation may have on the rate of oxidation of epinephrine under controlled conditions. The initial oxidative step is shown to be the oxidation of the sulfite ion. At a critical sulfite ion concentration, adrenochrome commences to form; however, the residual sulfite apparently reacts with the adrenochrome, resulting in a colorless adrenochrome sulfonate. It continues to form until all the sulfite is consumed, whereupon the solution deteriorates with visible discoloration. In the dark, epinephrine will remain stable to oxidation until light catalyzed, even after the destruction of all the sulfite and the formation of the adrenochrome sulfonate.

IT HAS BEEN reported recently by the authors (1) that boric acid stabilizes epinephrine against sulfite attack in an oxygen-free atmosphere. Epinephrine reacts with boric acid forming a boro-epinephrine chelate in accordance with the reaction given previously (1). The stability constant for the reaction was found to be 2×10^{-5}; however, use of an excess of boric acid and neutral or slightly alkaline pH conditions converts the vast majority of the epinephrine into the chelate form. The chelate form was found to be stable to sulfite attack. Since these conditions maintain stability of the epinephrine in the package until opened, it remains to investigate the effect of these additives on the stability of the epinephrine under oxidative attack.

The oxidation of epinephrine has been studied by many workers and has recently been reviewed by Heacock (2). At intermediate pH, epinephrine in aqueous buffered solution is oxidized to red substances; the speed of the reaction and the nature of the final products are dependent on the catalysts and buffers employed. In general, oxygen uptake begins only after a considerable induction period. Trace metals, particularly copper, manganese, and nickel, have been shown to initiate the reaction, possibly by the formation of an autoöxidizable epinephrine-metal chelate (3–5). Once the reaction is initiated, adrenochrome becomes a significant factor in the overall reaction rate (6, 7). Iron and copper chelating agents such as EDTA have been shown to increase the rate of oxidation, and other chelating agents also have been shown to increase the oxidation rate (8, 9). The catalytic action of adrenochrome has been postulated as being due to a chelated form of the compound (6). Trautner and Messer (10) reported on the effect of boric acid on the oxygen uptake of epinephrine and reported a stabilizing effect on epinephrine in alkaline solution.

Reducing agents such as sodium bisulfite (11), ascorbic acid, cysteine, and other reducing and

Received April 28, 1961, from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Accepted for publication July 6, 1961.
Supported in part by a grant-in-aid from the Barnes-Hind Laboratories, Sunnyvale, Calif.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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Reducing agents such as sodium bisulfite (11), ascorbic acid, cysteine, and other reducing and

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chelating agents (12) have been shown to inhibit the oxidation of epinephrine by molecular oxygen. Among these agents, sodium bisulfite appears to be the most commonly used antioxidant.

The oxidation of sulfite ions by oxygen has been shown to be a chain reaction, very sensitive to both positive and negative catalysts. One of the classical papers on this subject is the work of Fuller and Chryst in 1941 (13). These workers found that extreme precautions had to be taken to prevent metal contamination of their system. Double distilled water stored in hard-glass bottles was found to introduce spurious results after storage for longer than 3 hours. The rate of oxidation was affected by the rate of stirring and an adequate rate of oxygen absorption could not be maintained in solutions containing more than 0.015 *M* sulfite. The rate of cupric ion catalysis was found to be 10^9-fold the uncatalyzed rate under their conditions. Mannitol acted as a negative catalyst. The rate of oxidation is dependent primarily on the sulfite ion concentration, and bisulfite was much less easily oxidized. Later workers have confirmed the basic facts of the studies of Fuller. A detailed mechanism for the autoxidation of sulfite ions has been proposed by Abel (14, 15).

EXPERIMENTAL

Materials and Solution Preparation.—No special purification of distilled water, glassware, or of the reagent grade chemicals was undertaken. A single source of each chemical was used throughout. Efforts were taken to standardize the procedures used in the preparation of the solution to avoid the accidental incorporation of chance contaminants. Therefore, it is possible that the solutions were reproducible in respect to contamination and reflect best commercial manufacturing techniques.

Oxidative Method A.—Solutions containing 0.082 *M* epinephrine hydrochloride and varying amounts of sodium bisulfite and boric acid were adjusted to pH 6.5. A 25-ml. quantity of solution was placed in 200-ml. multiple-dose vials and attached to a reciprocating shaking device in a constant temperature bath at 25°. The solutions were open to air and exposed to light during the day from overhead fluorescent light, from a distance of approximately 10 feet. Samples were taken at the time of appearance of the visible red coloration of the solutions and at appropriate intervals thereafter. The solutions were examined for their absorbance at 440, 480, 550, and 600 mμ.

Oxidative Method B.—A fluorescent desk lamp containing two 20-inch fluorescent G. E. daylight tubes was inverted so that the light was reflected upward. Two polymethacrylate plates were separately spaced above the lights to allow air circulation. Glass beakers were thereby placed 2.5 inches from the light source. A Weston photometer was used to select that portion of the fluorescent tube which yielded uniform light intensity. Approximately the middle two-thirds was used.

Solutions containing sodium bisulfite and epinephrine, with or without boric acid, were studied alone and in combination. Air was bubbled through a coarse sintered-glass gas dispersion tube after water saturation and in a manner that the gas flow was essentially equal in all tubes. The rate of gas flow was as rapid as possible without dispelling a portion of the contents from the containers. In several determinations, the solutions were also stirred at 1750 r.p.m. by a two-bladed propeller. Certain solutions were covered with aluminum foil to exclude light, yet allowing other conditions of the experiment to remain unchanged.

The solutions were assayed for their sulfite content by iodometric titration and for their epinephrine deterioration products by spectrophotometric methods.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical determination utilizing oxidative method B. These solutions contained 0.1% sodium bisulfite and were adjusted to pH with standard sodium hydroxide. Since the conditions for the reaction were not as ideal as used by Fuller, the curves were not exactly duplicated on repetition. However, the variation was of a minor qualitative nature. Curve A illustrates the rate of oxidation observed with the sodium bisulfite solutions buffered to pH 7.0 with boric acid and sodium hydroxide. The half-life of the reaction was observed to be 8 to 9 minutes in a series of five duplicate determinations. Curve B illustrates the results in the absence of boric

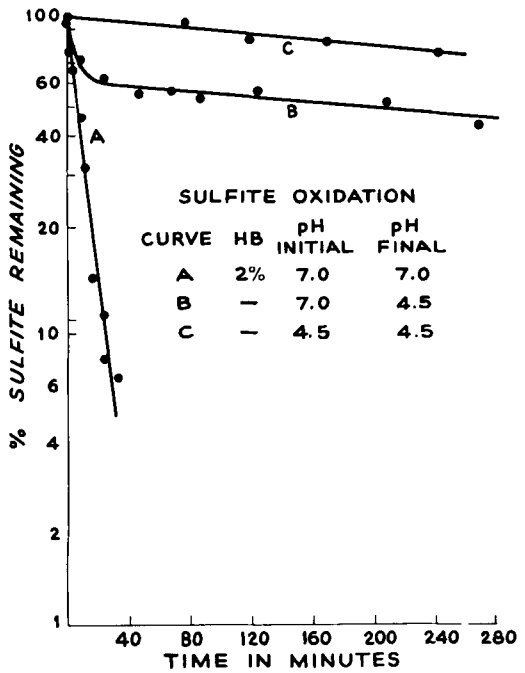


Fig. 1.—Oxidation of 0.1% sodium bisulfite solution at various initial hydrogen ion concentrations in the presence and absence of boric acid (HB). Data were obtained by oxidative method B.

acid buffer. Under these conditions, the oxidation of sulfite to sulfate ion increases the acidity of the solution. At pH 4.5, virtually all the sulfite ion vanishes and the oxidation rate is dependent on the remaining bisulfite ion concentration. This is verified by the data represented in curve C where the initial pH is adjusted to 4.5 and the rate of oxidation follows the linear portion of curve B.

As is well recognized, the first visible product of the oxidation of epinephrine is the formation of the red adrenochrome, which has a broad absorption maximum at 480 mμ. The onset and relative rate of oxidation can, therefore, be detected spectrophotometrically. The effect of sulfite and boric acid concentration on the rate of oxidation of epinephrine (method A) buffered at pH 6.5 is shown in Fig. 2. Two groups of curves are presented, all containing 1.5% (0.082 *M*) epinephrine. A comparison of the 0.2% to the 0.4% sodium bisulfite series indicates that the induction period is not doubled by a doubling of the concentration of the bisulfite. A solution containing 2.0% boric acid and 0.2% bisulfite has an induction period of 51 hours *vs.* 68 hours at the 0.4% level. An increase in concentration of boric acid at a fixed pH of 6.5 appears to accelerate the rate of oxidation of the bisulfite and therefore influences the length of the induction period. This may be due to trace impurities contained in the boric acid. Within experimental error, the slopes of the curves are identical, indicating that the rate of oxidation of the epinephrine is constant after the exhaustion of the antioxidant. An increase in concentration of boric acid will increase proportionately the concentration of the boro-epinephrine chelate, as is apparent from the reaction given by Eq. 1. This chelate does not influence the oxidative process under the present experimental conditions, possibly due to the presence of trace metals which form a much stronger chelate.

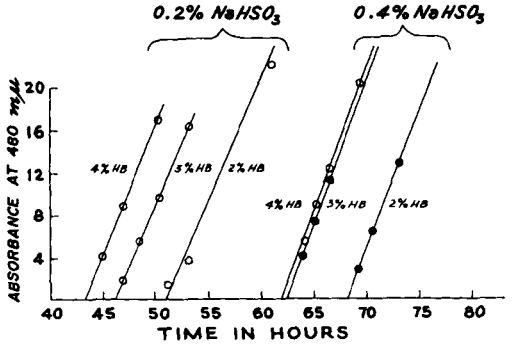


Fig. 2.—Oxidative studies on 1.5% epinephrine solutions at pH 6.5 by oxidative method A.

In the course of the assay of epinephrine solutions exposed to oxygen in the presence of bisulfite and boric acid buffer, a secondary ultraviolet absorption peak was observed at 350 mμ, increasing in intensity just prior to the development of the visible adrenochrome discoloration. In a recent study, van Espen (16), reported the formation of an adrenochrome bisulfite addition product, and presented detailed evidence indicating that this product is an adrenochrome sulfonate with the sulfonate group attached at position 4 or 7. The absorption spectrum of the

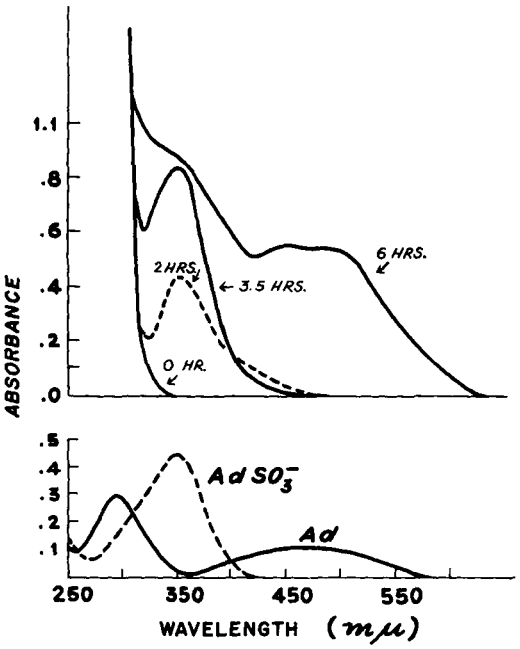


Fig. 3.—Upper curves: absorption spectra of epinephrine-bisulfite-boric acid solution at various intervals during the oxidation by method B. The curves were obtained from solution in a 3.0-mm. path length. Lower curves: absorption spectra of adrenochrome sulfonate ($AdSO_3^-$) and adrenochrome (Ad), redrawn from van Espen (16). Concentration = 2.8×10^{-5} *M*/L.; 1.0-cm. path length.

isolated compound was reported, and is redrawn in Fig. 3. The upper curves of Fig. 3 indicate the typical results obtained in spectrophotometric analysis of the epinephrine solutions. Prior to the exposure to oxygen by method B, the undiluted solution shows very strong absorption below 315 mμ due to the high concentration of epinephrine. Figure 3 includes the absorption curves for the same solution after 2, 3.5, and 6 hours. At the earlier periods, the solution contains bisulfite ions and is colorless to the eye; however, the absorption peak at 350 mμ indicates the presence of an oxidative product of epinephrine, probably the adrenochrome sulfonate, reported by van Espen. The 6-hour curves illustrates the results obtained after the oxidative reaction has exhausted all of the antioxidant. The curve indicates the presence of the adrenochrome sulfonate peak at 350 mμ, a considerable portion of adrenochrome since there is a peak with a maximum at approximately 480 mμ, and also the presence of higher oxidation products due to the secondary maximum at approximately 435 mμ.

Figure 4 presents a detailed experiment conducted by oxidative method B, utilizing a 1.5% epinephrine solution buffered at pH 7.0 including 2% boric acid and 1.0% sodium bisulfite. The rate of oxidation of the sulfite was followed iodometrically and the results are reported as the curve drawn through the closed circles. Under the conditions, the rate of oxidation is first order in respect to the bisulfite concentration. Even though the experimental conditions duplicated those used in the data presented in Fig. 1, the half-life for the initial portion of the reac-

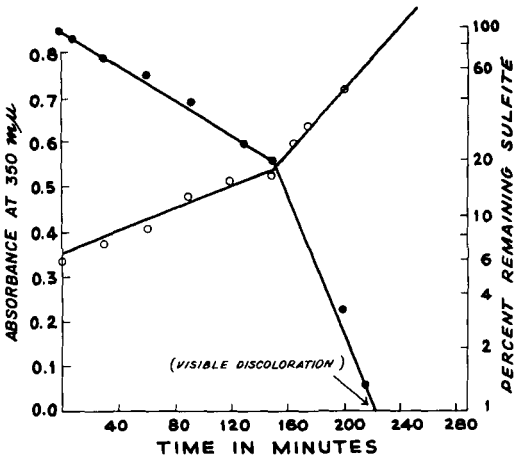


Fig. 4.—Illustrating the rate of oxidation of sulfite ions in the presence of 1.5% epinephrine and 2.0% boric acid at pH 7.0 (closed circles), and the rate of formation of adrenochrome sulfonate as measured by the absorbance at 350 mμ (open circles).

tion is approximately 65 minutes, while the identical solution in the absence of epinephrine possessed a half-life of 8 to 9 minutes. Catechol derivatives are known to be strong chelating agents (17, 18) for cupric and many other metallic ions, and it might be proposed that the epinephrine is a stabilizing agent for the sulfite by chelation of the trace metals.

It was interesting to note the marked change in rate of oxidation of the sulfite near the end of the reaction, even though the pH of the solution remained essentially constant. This second curve was duplicated repeatedly and could be followed to the last traces of sulfite in the solution. The half-life for the second reaction was roughly approximated to be 16 minutes. In a number of the experiments, the second portion was nonlinear. Simultaneous with the analysis of the sulfite content of the solutions, samples were examined spectrophotometrically at 350 mμ to obtain a more definitive understanding of the rate of appearance of the postulated adrenochrome sulfonate compound. The results are reported in Fig. 4 (open circles), and give a clear indication of the events taking place. Apparently there is a small progressive oxidation of a small number of the epinephrine molecules to adrenochrome sulfonate throughout the early part of the reaction, even though the sulfite is present in the solution. When the concentration of the bisulfite falls to approximately 20% of the original value, the rate of appearance of the adrenochrome sulfonate increases rapidly and the sulfite ions are withdrawn from the solution by two processes: (*a*) oxidation of sulfite, and (*b*) reaction with adrenochrome with the formation of adrenochrome sulfonate. The data of van Espen allows one to estimate the molar extinction coefficient of the adrenochrome bisulfite to be approximately 15,000. The increased rate of disappearance of sulfite in the second portion of the reaction cannot be accounted for completely on the basis of reaction with the adrenochrome. Therefore, an increase in the direct oxidative reaction must be presumed.

It must be emphasized that all of the oxidative studies of epinephrine reported above have been conducted under direct (method B) and diffuse (method A) exposure to fluorescent light. When identical solutions were run in the dark by either method, the rate of oxidation of the sulfite was represented by a constant rate of oxidation similar to the first portion of the bisulfite curve in Fig. 4. However, when all of the bisulfite is oxidized, epinephrine in the solution does not immediately oxidize. A long induction period follows which may be continued for days. Immediately after exposure to light, these solutions commence to oxidize and turn red.

It seems apparent, therefore, that some component in the solution is photochemically active in initiating the reaction. Since the 2-hour and 3.5-hour curves of Fig. 3 indicate the presence of an absorbing compound, believed to be adrenochrome sulfonate, it is postulated that this compound is the photochemically active molecule which catalyzes the initial stage in the epinephrine deterioration in the presence of sodium bisulfite. When the last traces of sodium bisulfite disappear, adrenochrome is no longer transformed to the colorless, but photochemically active sulfonate. Since adrenochrome absorbs in the visible, it can be postulated that higher light intensity in this region favors the catalysis via the adrenochrome.

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Mechanism of Phenobarbital Degradation

By FREDERICK TISHLER, JOSEPH E. SINSHEIMER, and JERE E. GOYAN

Previous studies on the degradation of barbiturates have indicated that the products of hydrolysis of the ionic and nonionic forms might be different. During the course of a study on the analysis of phenobarbital it was discovered that one of the postulated products (phenylethylacetylurea) could be determined in the presence of phenobarbital. Experiments were therefore undertaken to follow the breakdown of phenobarbital and the ureide at different pH values. The pseudo first-order rate constants for phenobarbital and the ureide were then determined. Analysis of equilibria phenomena and series first-order reaction kinetics show that the fraction decomposing by the ureide path is dependent on the concentration of the ionic form as previously postulated.

PREVIOUS WORK on the degradation of barbiturates (1) led to the postulate that the mechanism of hydrolysis of the ionized and nonionized forms was different, leading to distinguishable intermediates[1] (Fig. 1). This postulate could be tested by determining the concentration of these intermediates as a function of varying concentrations of the ionic and nonionic forms. Unfortunately, the determination of these intermediates is difficult for most of the barbiturate series. During a study on the analysis of phenobarbital (2) it was found that both phenobarbital and the ureide (G) can be determined spectrophotometrically in the presence of the decomposition products of phenobarbital. This, therefore, makes it possible to test the postulated mechanism.

EXPERIMENTAL

Reagents and Apparatus.—Phenobarbital, U.S.P. grade was recrystallized from dilute ethanol, m.p. 176–178°. Phenylethylacetylurea was prepared by thermal degradation of a 5% aqueous solution of phenobarbital and isolation of the precipitate. The ureide was recrystallized from dilute ethanol, m.p. 147–149° (3–5).

Ammonia buffer solutions (0.2, 0.4, 0.6, and 0.8 total molarity NH_3 and NH_4^+) at pH values of 8.0, 8.5, 9.0, and 0.8 *M* at a pH of 8.2, 8.7, and 9.5 were used. The total ionic strength was adjusted to 1.0 with potassium chloride in each case. A Beckman model DU spectrophotometer and a Beckman zeromatic pH meter were used.

Assays.—The concentration of phenobarbital was followed by determining the change in absorbance at 241 mμ in the following manner: a 10-ml. sample was diluted to 100 ml. with ammonia buffer (pH 10.0) and two 10-ml. aliquots of this were taken. One of these was diluted to 100 ml. with the same ammonia buffer while the second sample was diluted with 10 ml. of 1.0 *N* hydrochloric acid and sufficient buffer to make 100 ml. (Final pH = 1.5.) This absorbance difference is now used to obtain the phenobarbital concentration free of interference by the breakdown products (2).

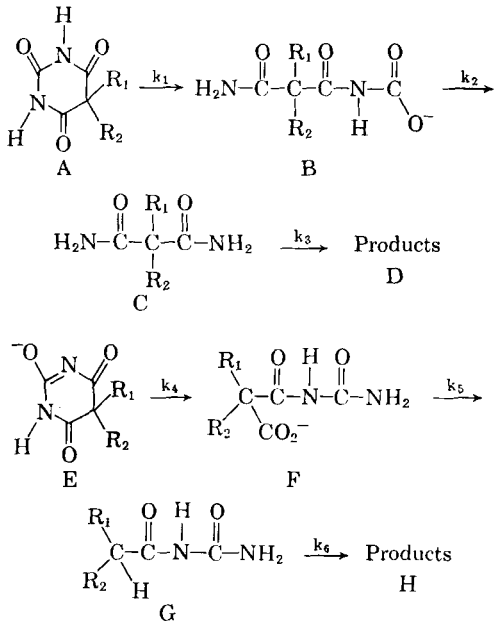


Fig. 1.—Degradation pathways of the nonionic and ionic species.

The concentration of phenylethylacetylurea during the degradation of phenobarbital was followed by measuring the absorbance of a 5-ml. sample at 245 and 260 mμ after the addition of 10 ml. of 1.0 *N* hydrochloric acid and dilution to 100 ml. with the pH 10.0 ammonia buffer. The use of simultaneous equations (after correction for the absorbance of phenobarbital) then made possible the determination of the ureide in the presence of diamide, or other interfering degradation products, all of which have approximately the same molar absorptivities at 245 and 260 mμ.

Concentration of Phenobarbital and Phenylethylacetylurea as a Function of Time.—Samples of phenobarbital (0.1% w/v final concentration) and

Received April 28, 1961, from the University of Michigan, College of Pharmacy, Ann Arbor.
Accepted for publication June 22, 1961.
Abstracted in part from a thesis by Frederick Tishler to be presented to the University of Michigan Graduate School in partial fulfillment of the requirements for the Ph.D. degree.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
The authors wish to thank Dr. Lee Worrell whose early interest in the analysis of barbiturates made this study possible.

[1] A different mechanism has been proposed by Hasegawa, *et al.* (7). Their mechanism, however, does not allow for the production of the diamide which has been isolated as a hydrolysis product (3, 8).

phenylethylacetylurea (0.01% w/v final concentration) were made up at different pH values using various concentrations of ammonium chloride for each pH value. (See Table I.) The samples were sealed in ampuls and kept in a constant temperature water bath at 60 ± 0.1°.

The reactions followed a pseudo first-order course since the log of the absorbance plotted *vs.* time was a straight line (Figs. 2 and 3). Table I gives the pseudo first-order rate constants for the different samples as determined by the method of least squares.

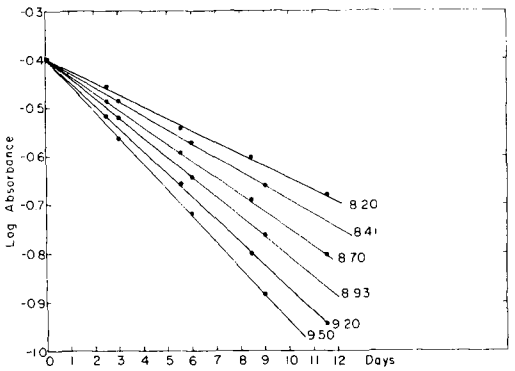


Fig. 2.—Degradation of phenobarbital at 60°. All at 0.8 *M*, numbers on individual lines are pH values.

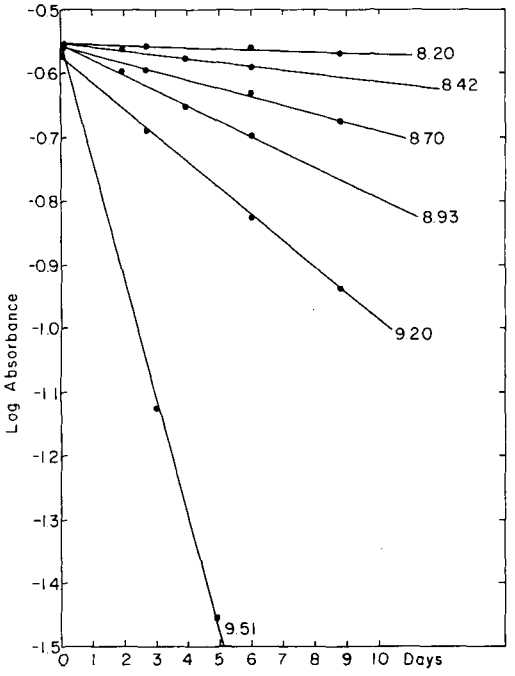


Fig. 3.—Degradation of phenylethylacetylurea at 60°. All at 0.8 *M*, numbers on individual lines are pH values.

DISCUSSION

In order to verify the similarity between the hydrolytic breakdown of phenobarbital and other barbiturates previously studied, a plot of the pseudo first-order rate constants *vs.* ammonium ion concentration was made (Fig. 4). These curves show a marked similarity to the curves obtained in previous work (1).

TABLE I.—FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF PHENOBARBITAL AND PHENYLETHYLACETYLUREA AT 60°

	Phenobarbital		
NH_4^+ *M*	pH at 25°	K, days^{-1}	Phenylethylacetylurea K, days^{-1}
0.1896	8.20	4.13×10^{-2}	...
0.3792	8.20	4.52×10^{-2}	...
0.5687	8.20	5.10×10^{-2}	...
0.7583	8.20	5.66×10^{-2}	4.03×10^{-3}
0.7030	8.42	6.70×10^{-2}	1.63×10^{-2}
0.1704	8.70	5.38×10^{-2}	...
0.3408	8.70	6.13×10^{-2}	...
0.5112	8.70	7.01×10^{-2}	...
0.6815	8.70	7.99×10^{-2}	3.10×10^{-2}
0.5569	8.93	9.29×10^{-2}	5.55×10^{-2}
0.1291	9.20	6.98×10^{-2}	...
0.2581	9.20	8.27×10^{-2}	...
0.3872	9.20	9.59×10^{-2}	...
0.5163	9.20	1.11×10^{-1}	9.55×10^{-2}
0.2922	9.51	1.23×10^{-1}	4.24×10^{-1}

The concentration of the ureide as a function of time may be predicted in the following manner (from Fig. 1)

$$E \xrightarrow{k_4} F \xrightarrow{k_5} G \xrightarrow{k_6} H$$

$$\frac{dE}{dt} = -k_4 [E], \quad \frac{dF}{dt} = -k_5 [F] + k_4 [E]$$

$$\frac{dG}{dt} = -k_6 [G] + k_5 [F]$$

Since species (F), the malonuric acid, is a very unstable compound (3, 5), it was assumed that $k_5 \gg k_4$ and the steady state approximation

$$-k_5 [F] + k_4 [E] = 0$$

or

$$[F] = \frac{k_4}{k_5} [E]$$

was made.

$$\therefore \frac{dG}{dt} = -k_6 [G] + k_4 [E]$$

This equation is solved in the usual manner (6) to obtain

$$[G]_T = \frac{E^0 k_4}{k_6 - k_4} [e^{-k_4 t} - e^{-k_6 t}] \quad \text{(Eq. 1)}$$

where E^0 is the original concentration of E.

If it is assumed that all of the barbiturate decomposes by the ureide path, E^0 becomes the original phenobarbital concentration, k_4 the pseudo first-order rate constant for the degradation of phenobarbital, and k_6 the pseudo first-order rate constant for the degradation of the ureide. The fraction of the total degradation going by the ureide path can then be defined as

$$f = \frac{[G]_M}{[G]_T} \quad \text{(Eq. 2)}$$

where $[G]_M$ is the measured concentration of ureide

TABLE II.—ANALYSIS OF FRACTION BY UREIDE PATH

pH	8.20		8.42		8.70		8.93		9.20		9.50	
Days	2.50	5.56	2.96	5.98	2.50	5.56	2.96	5.98	2.50	5.56	2.96	5.98
G_T, mg.	0.646	1.320	0.859	1.529	0.835	1.605	1.089	1.811	1.105	1.780	0.832	0.810
G_M	0.025	0.049	0.046	0.092	0.093	0.193	0.173	0.281	0.315	0.500	0.400	0.382
f	0.039	0.037	0.054	0.060	0.111	0.120	0.159	0.155	0.285	0.281	0.481	0.472
Av. f	0.038		0.057		0.116		0.157		0.283		0.477	

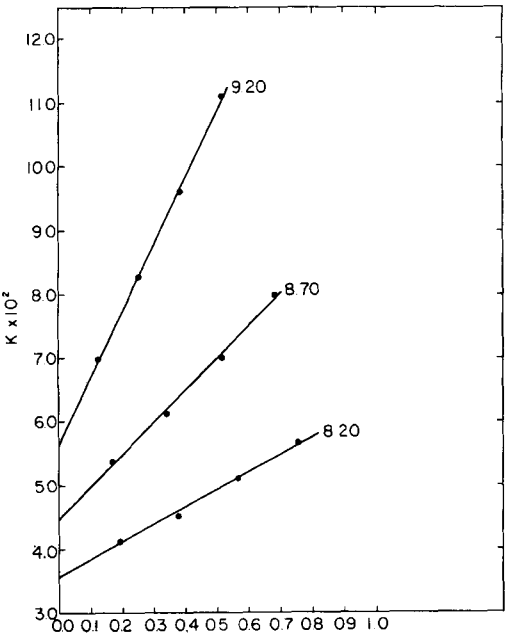


Fig. 4.—Pseudo first-order rate constants plotted *vs.* ammonium ion concentration at 60°. Numbers on individual lines are pH values.

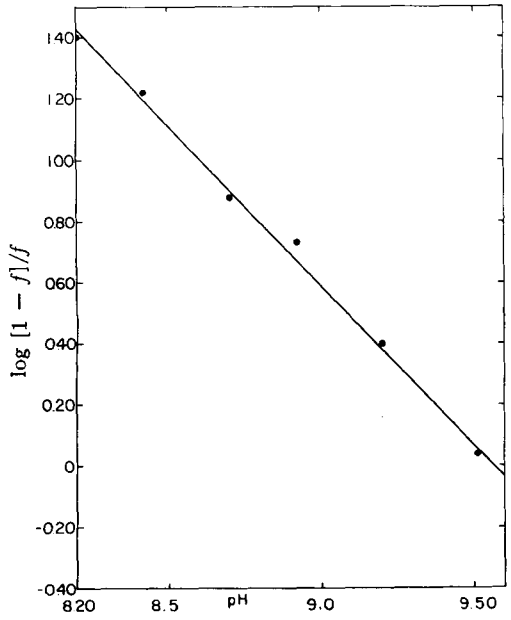


Fig. 5.—The effect of pH on fraction (f) degrading by ureide pathway.

at some given time and $[G]_T$ is the "theoretical" concentration computed by Eq. 1.

The assumption that the ureide pathway is taken by the ionized form of phenobarbital may now be formulated as follows: equilibrium requires

$$K_A = \frac{[E]\,[H^+]}{[A]} \qquad \text{(Eq. 3)}$$

where $[H^+]$ is the hydronium ion concentration of the solution

$$R_1 = k_1\,[A] \qquad \text{(Eq. 4)}$$

$$R_2 = k_4\,[E] \qquad \text{(Eq. 5)}$$

where R_1 and R_2 are the rates of degradation of the two forms, then

$$f = \frac{R_2}{R_1 + R_2} = \frac{k_4\,[E]}{k_1\,[A] + k_4\,[E]} = \frac{k_4\,[K_A]}{k_1[H^+] + k_4[K_A]} \qquad \text{(Eq. 6)}$$

and

$$1/f = 1 + \frac{k_1}{k_4\,K_A}\,[H^+] = 1 + \alpha\,[H^+] \qquad \text{(Eq. 7)}$$

or

$$\log\left[\frac{1-f}{f}\right] = \log\alpha - \text{pH} \qquad \text{(Eq. 8)}$$

Equation 8 therefore predicts that if the proposed mechanism is correct, a plot of log $[1 - f]/f$ *vs.* pH should be a straight line with a slope of -1. The experimental results are summarized in Table II and Fig. 5. The least squares slope of the line in Fig. 5 is -1.035. Thus, it appears that the proposed mechanism fits the experimental facts.

SUMMARY

1. The degradation of phenobarbital and phenylethylacetylurea have been followed concurrently in solution.

2. The results of these studies have been used to corroborate previous hypotheses about the degradation mechanism of barbiturates.

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Studies on *Vinca major* (*Apocynaceae*) II

Phytochemical Investigation

By N. R. FARNSWORTH, H. H. S. FONG, R. N. BLOMSTER†, and F. J. DRAUS‡

Vinca major L. (*Apocynaceae*), commonly known as "Pervenche Grande" or the "Greater Periwinkle," has been mentioned frequently as a folk remedy. This plant, collected in Yugoslavia, has been subjected to a phytochemical screening and found to contain alkaloids, saponins, unsaturated sterols, organic acids, and phenols. Tannins, flavanols, and cardiac glycosides were not detected by the micromethods used. The results of a proximate analysis are also reported together with a method for the chromatographic separation of the total alkaloids. Paper partition and thin layer chromatographic data of the separated fractions are presented to indicate the complex character of the total alkaloids of this plant. An aqueous extract of the leaves and stems, as well as the total alkaloids, were shown to have no leukopenic activity in rats.

VINCA MAJOR[1] has been used for centuries as a folk remedy in the treatment of diabetes (2), as an abortifacient (2, 3), vulnerary, and in the treatment of menorrhagia (2). The plant has a wide distribution, having been introduced to the United States (4), England, Portugal, Spain, Malta, and Algeria (1). It is reported indigenous to France, Italy, Switzerland, Sicily, Madeira, Canary Islands, Crete, Rhodes, Chios, and the south and east coasts of the Black Sea (1). There is a great deal of current interest in the plants of the genus *Vinca*, not only because they are members of the alkaloid-rich *Apocynaceae*, but because of the recent isolation of the antileukemic alkaloid vincaleukoblastine from a related plant, *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) (5).

To date, nine crystalline alkaloids have been isolated from *V. major* (Table I). They include reserpinine (6), vincamajoridine (akuammine) (7), vincamajoreine (8), vincamajine (9), perivincine (10), pubescine, vinine, and an alkaloid with m.p. 194–195° (11), as well as an alkaloid thought to be serpinine (6). Djerassi, *et al.* (12), have suggested that serpinine is identical with tetraphyllicine. In addition to these alkaloids, ursolic acid (13), dambonitol (14), and robinoside (15) have been isolated, and *o*-pyrocatechuic acid has been found to be present in acid hydrolysates of this plant (16).

TABLE I.—ALKALOIDS FROM *Vinca major* L.

No.	Name	Formula
1	Reserpinine	$C_{22}H_{26}N_2O_4$
2	Vincamajoridine	$C_{22}H_{26}N_2O_4$
3	Vincamajoreine	$C_{21}H_{26-28}N_2O_2$
4	Vincamajine	$C_{22}H_{26}N_2O_3$
5	Perivincine	$C_{22}H_{28}N_2O_4$
6	Pubescine	$C_{20}H_{26}N_2O_4$
7	Vinine	$C_{19}H_{26}N_2O_4$
8	Alkaloid m.p. 194–195°	
9	Serpinine (?) m.p. 316°	

The total alkaloids of *V. major* have been found to be ganglioplegics as well as spasmolytics, but only exert a transient hypotensive effect (17). Reserpinine (18) and perivincine (10) have been reported to produce only a transient hypotensive action in animals but vinine exerts a pronounced and prolonged blood pressure depression (11).

In this communication we wish to report the results of a phytochemical study of the leaves and stems of *V. major* and the effects of extracts of this plant on the leukocyte counts of rats.

EXPERIMENTAL

Phytochemical Screening.—The plant material used in this investigation was collected in Yugoslavia during the summer of 1957 and consisted of the dried leaves and stems.[2] The air-dried material was ground to a coarse powder and screened for alkaloids, saponins, flavanols, tannins, unsaturated sterols, organic acids, and phenols by the micromethod of Wall, *et al.* (19). In addition, cardiac glycosides were tested for by the method of Krider, *et al.* (20). The results of these tests are listed in Table II. There is some conflict of these results with those obtained by Wall, *et al.* (21), who used *V. major* leaves and twigs collected in Athens, Georgia, during 1951 and recorded negative alkaloid, organic acid, and phenol tests for this plant. The

Received April 28, 1961, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh 13, Pa.
Accepted for publication June 16, 1961.
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This investigation was supported, in part, by grants from the Health Research and Services Foundation, Pittsburgh, Pa., and the American Cancer Society.
[1] Synonymous with *Vinca major* L. var. *major* Pich., *V. pubescens* Urv., *V. grandiflora* Salisb., *V. ovatifolia* Stokes, and *V. obliqua* Porta (1).
[2] Generously supplied as botanically authenticated *Vinca major* L. leaves and stems by the S. B. Penick & Co., Inc., 100 Church Street, New York 8, N. Y.

TABLE II.—PHYTOCHEMICAL SCREENING

Alkaloids	+
Saponins	+
Sterols, unsaturated	+
Organic acids	+
Phenols	+
Flavanols	−
Tannins	−
Cardiac glycosides	−

negative alkaloid test by these workers can be explained on the basis of their extracts being too dilute. Their current revised alkaloid testing procedure has been modified so that instead of 1 ml. being equivalent to 0.2 Gm. of dry plant sample, it is now equivalent to 4.0 Gm. (22). The positive hemolysis test for saponins is due to the ursolic acid content of the leaves.

Proximate Analysis.—A proximate analysis of the air-dried leaves and stems of *V. major* was conducted according to standard U.S.P. and A.O.A.C. methods (23–30). The results are shown in Table III and represent averages for four determinations expressed in terms of the air-dried material.

TABLE III.—PROXIMATE ANALYSIS

Determination	Per Cent[a]	Method (Ref.)
Total ash	3.76	(23)
Acid-insoluble ash	1.03	(23)
Crude fiber	10.49	(23)
Moisture		
By toluene distillation	6.99	(24)
By method for drugs containing no constituents volatile at 100°	6.74	(24)
Extractives		
Alcohol-soluble	36.31	(25)
Diluted alcohol-soluble	23.07	(25)
Petroleum ether-soluble	2.75	(25)
Volatile ether-soluble	0.73	(25)
Nonvolatile ether-soluble	4.96	(25)
Water-soluble	22.07	(26)
Starch	3.72	(27)
Pentosans	12.54	(28)
Reducing sugars	13.72	(29)
Organic and ammoniacal nitrogen	1.39	(30)

[a] Average per cent for four determinations.

Alkaloid Isolation and Fractionation.—The method used for the isolation of total alkaloids and separation into crude fractions I, II, and III has been previously reported (10). Figures 1 and 2 represent schematic outlines of this work.

Chromatographic Separation of Alkaloid Fractions.—*Fraction I.*—Three grams of fraction I (petroleum ether-insoluble) crude alkaloids was dissolved in 10 ml. of methanol with the aid of gentle heat. Fisher adsorption alumina, 10 Gm. (80–200 mesh, pH 9.95), which had been previously heated at 200° for 4 hours, was added to the solution of alkaloids and the mixture stirred on a water bath until the methanol was removed. A glass column (36 × 3/4 in.) was tightly packed with 230 Gm. of alumina, activated as described, and the alkaloid-adsorbed alumina added to the column in the same manner. A small pledget of glass wool was added to prevent disruption of the adsorbent on addition of the eluent. Elution of the alkaloids was effected by means of the following series of gradient eluents: benzene, benzene-methanol (99.5:0.5), benzene-methanol (99:1), benzene-methanol (98:2), benzene-methanol (95:5), benzene-methanol (90:10), benzene-methanol (50:50), and methanol.

Fractions were collected utilizing an automatic fraction collector equipped with a 20-ml. siphon.[3] Each fifth fraction was tested for alkaloids in the usual manner with Mayer's reagent. By observing the density of precipitation with this reagent it was possible to follow the relative concentration of alkaloids throughout the separation. Consecutive fractions were pooled according to the results of this estimation, evaporated to dryness, and weighed. When negative fractions could not be found for cut-off points, the low point between any two peaks was selected. The results of the separation of fraction I crude alkaloids are shown in Table IV.

TABLE IV.—CHROMATOGRAPHY OF FRACTION I (3 GM.)

Fraction No.	Volume, ml.	Eluent	Weight, Gm.	Test for Alkaloids
I-A	2,500	Benzene	...	..
I-B	2,000	Benzene-methanol (99.5–0.5)	...	..
I-C	2,000	Benzene-methanol (99:1)	0.420	+
I-D	2,100	Benzene-methanol (98:2)	0.249	+
I-E	2,000	Benzene-methanol (95:5)	0.223	+
I-F	1,500	Benzene-methanol (90:10)	0.119	+
I-G	2,000	Benzene-methanol (50:50)	0.132	+
I-H	3,600	Methanol	0.245	+

Fraction II.—This fraction (benzene-petroleum ether-soluble) of alkaloids was treated in the same manner as fraction I except that 4 Gm. of crude alkaloids was dissolved in 10 ml. of chloroform and mixed with 10 Gm. of alumina prior to application on a 230-Gm. column of alumina, as previously described. The same series of gradient eluents was used (Table V).

Fraction III.—Four grams of fraction III (benzene-insoluble) crude alkaloids was extracted with five successive 20-ml. portions of methanol by trituration in a mortar, followed by filtration. The nonalkaloidal residue weighed 1 Gm. The methanol filtrates containing 3 Gm. of crude alkaloids were mixed with 23 Gm. of alumina and dried as previously described. This mixture was then placed on top of a packed column containing 200 Gm. of alumina and the same series of eluents added. The results are summarized in Table VI.

[3] Rinco chromatographic fraction collector, Rinco Instrument Co., Greenville, Ill.

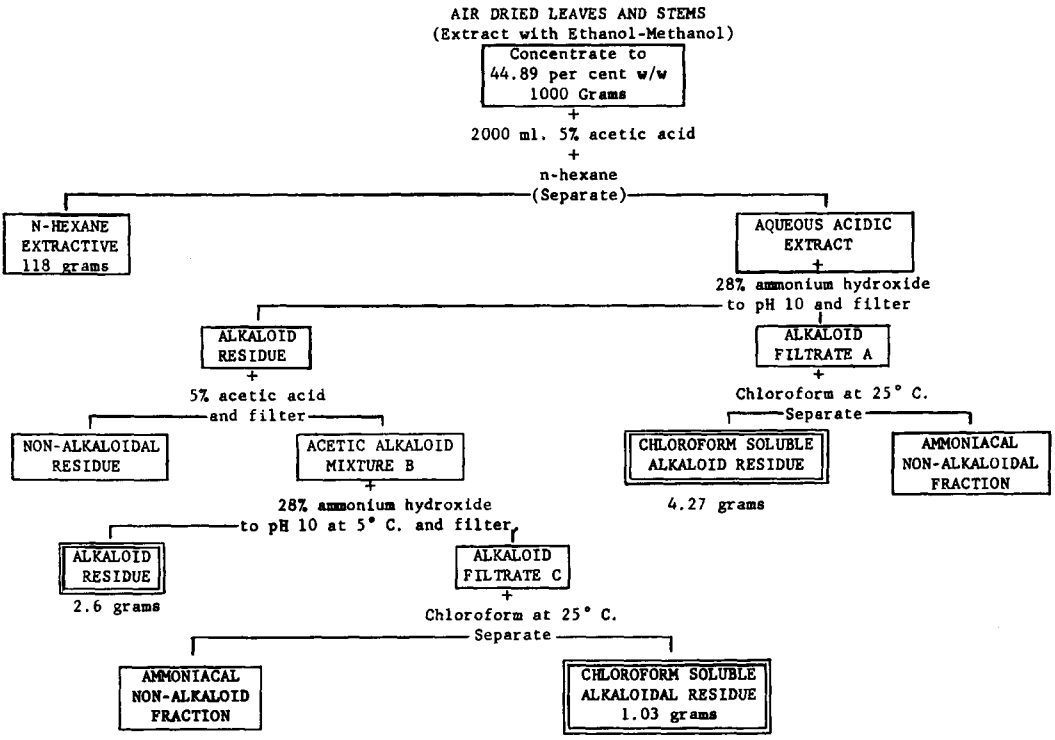


Fig. 1.—Flowsheet for the removal of the total crude alkaloids from the leaves and stems of *Vinca major*.

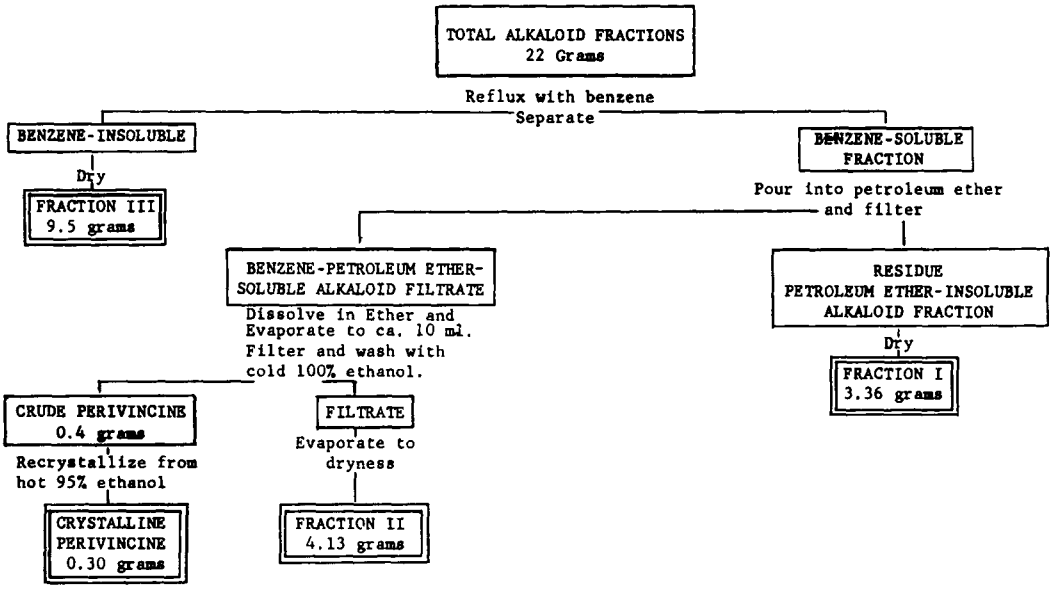


Fig. 2.—Flowsheet for the preliminary fractionation of the total crude alkaloids from the leaves and stems of *Vinca major*.

Alkaloid Composition of Separated Fractions.—The alkaloid composition of the separated fractions was determined by paper as well as by thin-layer chromatography. It was possible to determine alkaloid spill-over in this manner by the comparison of R_f values as well as by the color and intensity of the alkaloid spots, as viewed under ultraviolet illumination, and by the use of Dragendorff's reagent.

Paper Partition Chromatography.—The most efficient paper chromatographic method for the separation of *Vinca* alkaloids was found to be that of Korzun, *et al.* (31), with the modification of pH adjustment of the formamide with formic acid to

TABLE V.—CHROMATOGRAPHY OF FRACTION II (4 GM.)

Fraction No.	Volume, ml.	Eluent	Weight, Gm.	Test for Alkaloids
II-A	4,000	Benzene	0.193	+
II-B	2,200	Benzene-methanol (99.5-0.5)	1.823	+
II-C	900	Benzene-methanol (99:1)	0.173	+
II-D	2,040	Benzene-methanol (98:2)	0.370	+
II-E	1,700	Benzene-methanol (95:5)	0.239	+
II-F	1,280	Benzene-methanol (95:5)	0.051	+
II-G	2,100	Benzene-methanol (95:5)	0.069	+
II-H	3,400	Benzene-methanol (90:10)	0.191	+
II-J	1,900	Benzene-methanol (50:50)	0.142	+
II-K	2,760	Methanol	0.135	+

TABLE VI.—CHROMATOGRAPHY OF FRACTION III (3 GM.)

Fraction No.	Volume, ml.	Eluent	Weight, Gm.	Test for Alkaloids
III-A	1,300	Benzene	...	..
III-B	1,900	Benzene-methanol (99.5:0.5)	0.011	+
III-C	1,110	Benzene-methanol (99.5:0.5)	0.058	+
III-D	1,300	Benzene-methanol (99:1)	0.033	+
III-E	3,100	Benzene-methanol (98:2)	0.140	+
III-F	2,600	Benzene-methanol (95:5)	0.173	+
III-G	1,280	Benzene-methanol (95:5)	0.066	+
III-H	1,780	Benzene-methanol (95:5)	0.059	+
III-J	3,400	Benzene-methanol (90:10)	0.134	+
III-K	1,900	Benzene-methanol (50:50)	0.276	+
III-L	2,100	Methanol	0.324	+

either pH 5.2 or pH 8.0. Samples of commercial formamide were found to vary in pH from 10.1 to 11.7 which led to variation in results. Solutions of the alkaloid fractions were prepared in either methanol or chloroform at 1% (w/v) concentrations and 50 λ of each applied. An equilibration period of 4 hours was found necessary in order to obtain reproducible results. The completed chromatograms were dried at 90–95° for one-half hour and then observed under long and short wave ultraviolet light. Fluorescent areas were outlined and the colors noted. After spraying with a modified Dragendorff reagent (32), the R_f values were calculated. In some instances areas giving a positive Dragendorff reaction were not distinct and appeared to run together. In these instances, the more sensitive fluorescent areas which had been marked, aided in the differentiation of distinct areas for R_f calculation. The results of chromatographing all of the alkaloid fractions in a system utilizing formamide adjusted to pH 8.0 are listed in Table VII. The R_f values tabulated represent averages of duplicate samples of each fraction.

TABLE VII.—R_f VALUES FOR SEPARATED FRACTIONS BY PAPER PARTITION CHROMATOGRAPHY AT pH 8.0[a]

	Fraction I	Fraction II	Fraction III
A	...	0 o	...
	...	0.32b	...
	...	0.70y	...
	...	0.85oy	...
B	...	0 b	0 b
	...	0.15b	...
	...	0.22b	...
	...	0.30b	...
	...	0.37b	...
	...	0.49b	...
	...	0.77b	...
C	0 o	0 y	0 b
	0.03b	0.08g	0.09o
	0.17o	0.16y	0.16b
	0.33y	0.26b	0.27y
	0.45p	0.32b	0.37b
	0.55b	0.41y	0.49b
D	0 b	0 y	0 y
	0.07b	0.06g	0.17y
	...	...	0.72y
E	0 b	0 y	0 y
	...	0.33y	0.07b
F	0 b	0 o	0 bb
	0.07b	...	...
G	0 b	0 g	0 b
	0.23b	...	0.20b
	0.38b	...	0.82b
	0.50b	...	...
	0.90b	...	...
H	0 b	0 b	0 b
	0.03b	0.29b	...
J	...	0 b	0 b
	...	0.23b	0.18b
	...	0.39b	...
	...	0.50b	...
	...	0.90b	...
K	...	0 b	0 b
	...	0.13b	...
	...	0.75y	...
L	...	...	0 b

[a] Each R_f represents the average from two chromatograms. Letters following each R_f refer to fluorescent color under long wave ultraviolet light: o = orange, b = blue, bb = brilliant blue, p = pink, y = yellow, g = green, oy = orange-yellow.

Thin-Layer Chromatography.—A rapid and relatively simple method for detecting the alkaloids in each fraction was thin-layer chromatography. Plates were prepared using silica gel G and the

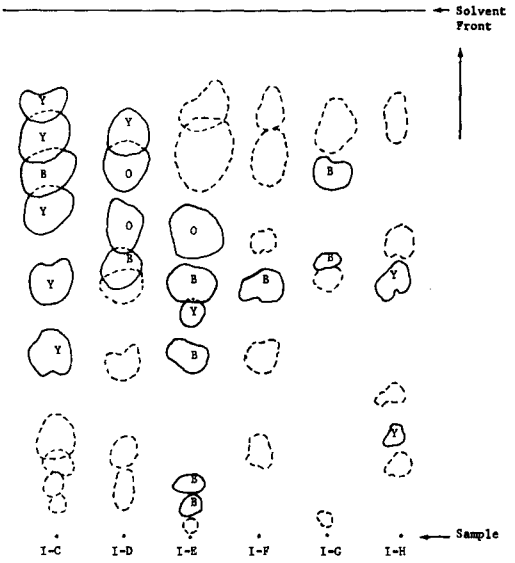


Fig. 3.—Thin-layer chromatogram of fraction I alkaloids using silica gel G adsorbent and chloroform-methanol (95:5) as eluent. Key: (under long wave U.V. light) B = blue, O = orange, Y = yellow fluorescence.

Desaga[4] apparatus. The adsorbent was mixed by slowly adding 40 ml. of distilled water, with stirring, to 30 Gm. of silica gel G. An additional 20 ml. of distilled water was then added, mixed thoroughly, and poured into the spreading applicator and applied to 200 × 200-mm. glass plates in the usual manner. The glass plates were then placed in a preheated oven at 105° for 30 minutes, allowed to cool to room temperature, and then spotted with the various alkaloid fractions. Solutions of the alkaloid fractions were prepared at 10% (w/v) concentrations in either methanol or chloroform. Ten-lambda increments were spotted on the prepared plates, allowed to air-dry, and then the plates were placed into equilibrated glass chambers lined with filter paper. Several eluents were used, with chloroform-methanol (95:5) giving the best separations. Normal development time was found to be about 1 hour. The results of the chromatographic separations using this method are shown in Figs. 3–5. Interpretation of the chromatograms was accomplished as previously described. The relative concentration of alkaloids, as indicated by the intensity of fluorescence under ultraviolet illumination, has been shown in the figures as follows: (*a*) spots of definite color and intensity are circled with a solid line enclosing a letter indicating the fluorescent color, (*b*) spots which could definitely be determined but were of low intensity so that colors could not be assigned to them are circled with broken lines.

Interpretation of Chromatograms.—The results of both the paper partition and the thin-layer methods of chromatography were compared to chromatograms of the following alkaloids:[5] reserpinine, yohimbine, α-yohimbine, lochnerine, vincamine, sarpagine, serpentine, vincamajoridine, ajmaline, ajmalicine, vincamajine, perivincine, reserpiline, tetrahydroalstonine, reserpine, deserpidine, and rescinnamine. The presence of serpentine, vincamine, sarpagine, and tetrahydroalstonine is strongly suspected from these results and work is now in progress to evaluate all of the alkaloid spots against a large number of reference alkaloids from the *Apocynaceae*. Although there is little doubt from our data that perhaps 40 or more distinct alkaloids are present in this plant, more work must be conducted to determine the extent of alkaloid spill-over from one fraction to another.

Screening of Extracts for Leukopenic Activity.—Noble, *et al.* (5), were the first to report on the leukopenic activity of *Vinca rosea* in experimental animals. Their study resulted in the isolation of vincaleukoblastine, the alkaloid responsible for the major portion of the leukopenic activity of crude extracts of *V. rosea*. Vincaleukoblastine is now available as an aid in the treatment of Hodgkin's disease and choriocarcinoma in humans.[6] Although these workers indicated that extracts of *V. major* as well as *V. minor* did not affect the blood picture of rats in their studies, it seemed advisable to confirm these results with studies using *V. major* from Yugoslavia, especially since Noble, *et al.* (5), reported that *V. rosea* plants grown as annuals in England possessed only about one-fourth of the leukopenic activity of plants grown as perennials in the West Indies. For testing purposes, two extracts were prepared; extract A was essentially an aqueous concentrate, containing all of the alkaloids of the plant in addition to other water-soluble materials, while extract B was a propylene glycol solution of total alkaloids as free bases.

Preparation of Extract A.—A Soxhlet extraction apparatus was packed with 380 Gm. of powdered air-dried leaves and stems and continuously extracted with methanol until free from alkaloids. The methanol extract was cooled to room temperature, filtered, and concentrated *in vacuo* at 40° to *ca.* 500 ml., mixed with 500 ml. of distilled water, shaken with Celite filter aid, and filtered. The filtrate was again concentrated *in vacuo* to 225 ml. which, after filtration, resulted in an alcohol-free aqueous extract containing the equivalent of 1.7 Gm. of air-dried plant material in each 1 ml.

Preparation of Extract B.—A portion of extract A was treated with 28% ammonium hydroxide solution to pH 10 with constant stirring. The precipitated alkaloids were removed by several extractions with chloroform. The combined chloroform extracts were dried over sodium sulfate, filtered, and evaporated to dryness *in vacuo*. The alkaloid residue was dissolved in propylene glycol to make a solution containing 0.155 Gm. in each 1 ml.

Leukocyte Counting.—Doses of 8.25 ml./Kg. of extract A and 0.82 ml./Kg. of extract B were selected after toxicity tests indicated that these doses were the highest that could be tolerated with 100% survival of all animals injected. Both total and differential leukocyte counts were performed in the usual manner utilizing blood from the tail of male albino rats.[7] All counts were made on consecutive

[4] Desaga apparatus for thin-layer chromatography, Brinkmann Instruments Inc., Great Neck, Long Island, N. Y.

[5] The authors wish to thank the S. B. Penick & Co., Abbott Laboratories, Charles Pfizer & Co., Ciba Pharmaceutical Products Inc., and Eli Lilly and Co., for generous samples of alkaloids as well as Drs. M. M. Janot, N. A. Rubin, S. Goodwin, C. Djerassi, and A. Stoll.

[6] Velban, trademarked name of Eli Lilly & Co., Indianapolis, Ind., for vinblastine sulfate.

[7] Holtzman Co., Madison 4, Wis.

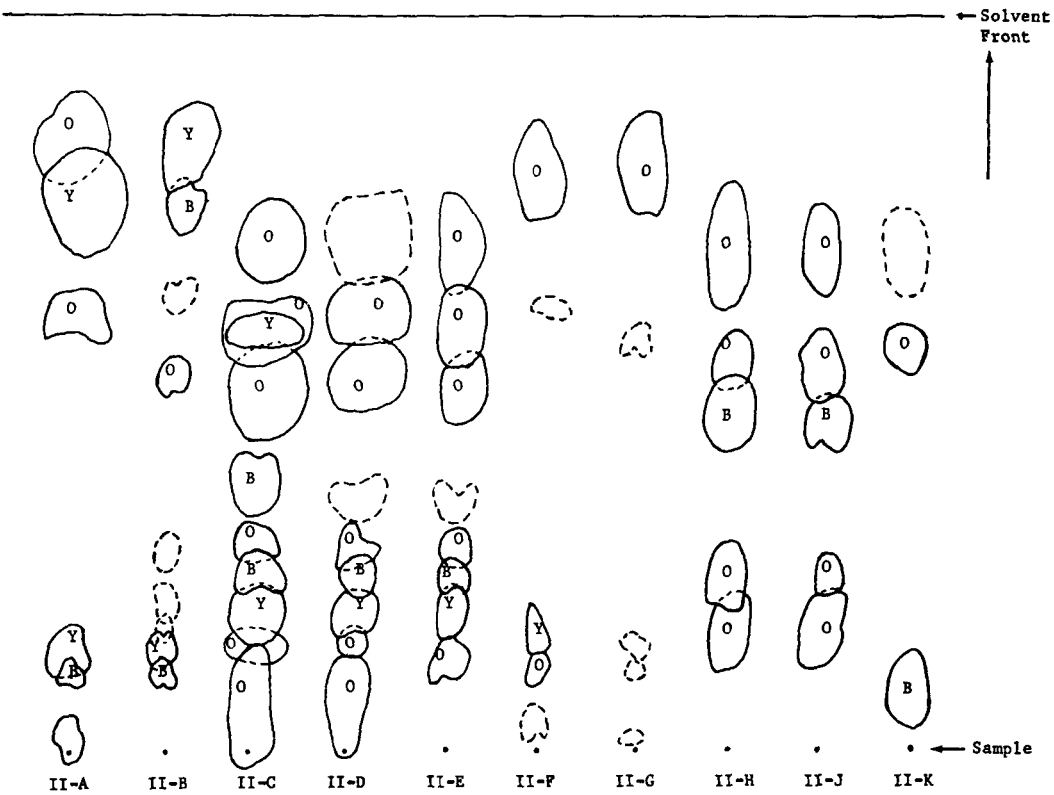


Fig. 4.—Thin-layer chromatogram of fraction II alkaloids using silica gel G adsorbent and chloroform-methanol (95:5) as eluent. Key: (under long wave U.V. light) B = blue, O = orange, Y = yellow fluorescence.

days and at approximately the same time each day. The rats were fed a normal diet *ad libitum*. The rats were divided into four groups of six animals each. In addition to daily blood counts, rat weight was tabulated since, according to Noble, *et al.* (5), leukopenia due to extracts of *V. rosea* was always accompanied by a drop in weight one to two days after i.p. injection. After 7 consecutive days of control normal blood counts from all animals, single i.p. injections were given. Group 1 received 8.25 ml./Kg. of extract A and group 2 received 8.25 ml. of distilled water as a control. Group 3 received 0.82 ml./Kg. of extract B, and group 4 received 0.82 ml./Kg. of propylene glycol as a control. Daily blood counts and weight recordings were continued for at least 6 days. The results as shown in Figs. 6 and 7 indicate that extract A and extract B do not depress the leukocyte counts under these experimental conditions. Differential counts in all groups remained within normal limits when compared to the control animals. These results are consistent with those reported by Noble, *et al.* (5), for their extracts of *V. major*.

DISCUSSION

This investigation has pointed out the complex nature of the total alkaloids of *V. major*. Through an interpretation of our chromatographic data, which will be reported at a later date, we can show the presence of at least 40 alkaloids in this plant. Although this is a rather large number it is not unusual since a related plant, *V. rosea*, has yielded at least 21 well described alkaloids at the time of this writing, in addition to four more which appear, from available data, to be additions to this list. *V. lancea* (*Catharanthus lanceus*) total alkaloids appear to include at least 70 separate alkaloids which we have been able to detect in some preliminary work by two-dimensional thin-layer chromatography. Although we have proposed that tetrahydroalstonine and sarpagine are present in the *V. major* alkaloids, it will be interesting, after additional work, to confirm this since these two alkaloids have not been reported to date as being present in any of the *Vinca* species which have been investigated, although they do appear in the *Catharanthus* group. Interestingly enough, of the 21 alkaloids isolated to date from species of *Catharanthus*, only vincamajoridine (akuammine) has been reported to be present in any of the *Vinca* species.

It has become evident to us during this investigation that thin-layer chromatography will prove to be an invaluable tool in phytochemistry and especially in chemotaxonomic studies. The method is simple, rapid, and accurate for the tentative identification of substances which are chromatographed on the same plate with known reference compounds.

The chromatographic separation of alkaloid fractions from *V. major* on a pilot scale has indicated that reasonable separation of alkaloids has been accomplished and that by inspection of paper and



Fig. 5.—Thin-layer chromatogram of fraction III alkaloids using silica gel G adsorbent and chloroform-methanol (95:5) as eluent. Key: (under long wave U.V. light) B = blue fluorescence, O = orange fluorescence, W = white fluorescence, Y = yellow fluorescence.

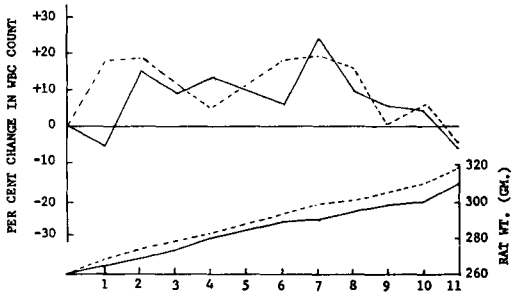


Fig. 6.—The effect of *Vinca major* extract A on rat wbc counts and weight after a single i.p. injection (8.25 ml./Kg.) *vs.* distilled water as a control. Mean values for groups of six rats following a 7-day control counting period. —— extract A, - - - - distilled water.

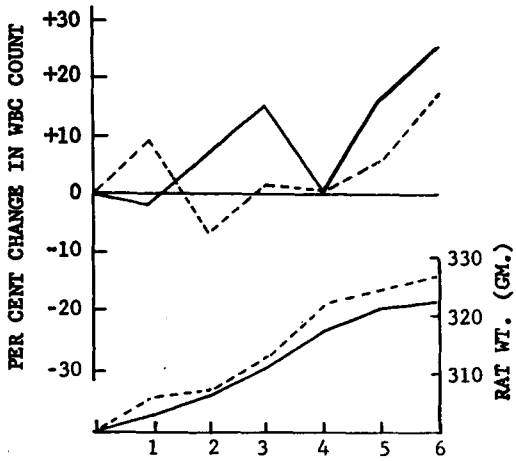


Fig. 7.—The effect of *Vinca major* extract B on rat wbc counts after a single i.p. injection (0.82 ml./Kg.) *vs.* propylene glycol as a control. Mean values for groups of six rats following a 7-day control counting period. —— extract B, - - - - propylene glycol.

thin-layer chromatograms, we can group certain eluates prior to attempting crystallizations from them.

SUMMARY

1. A phytochemical screening of the leaves and stems of *Vinca major* L. collected in Yugoslavia has shown the presence of alkaloids, saponins, unsaturated sterols, organic acids, and phenols. Tannins, flavanols, and cardiac glycosides were not detected.

2. A proximate analysis of the plant material is reported.

3. Methods for the separation of total alkaloids as well as their fractionation by gradient elution chromatography on alumina and detec-

tion by paper partition and thin-layer chromatography are described.

4. Screening of an aqueous extract as well as the total alkaloids of *V. major* in rats for possible leukopenic activity has confirmed a previous report of the absence of such activity.

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Stability of Antibacterial Preservatives in Parenteral Solutions I

Factors Influencing the Loss of Antimicrobial Agents from Solutions in Rubber-Stoppered Containers

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Losses of preservative due to degradation and absorption in rubber-closed multiple-dose vials were investigated. The preservatives evaluated were benzyl alcohol, phenylethyl alcohol, *p*-chloro-β-phenylethyl alcohol, chlorobutanol, and methylparaben. The elastomer closures used in this study were intended to be representative of those most commonly employed for pharmaceuticals and were natural, neoprene, and butyl rubbers. The temperature dependency of the degradation and diffusion processes were studied. An attempt was made to correlate preservative loss and microbiological activity.

RUBBER CLOSURES are used extensively by pharmaceutical manufacturers to seal vials containing injectable solutions. It is well known that rubber may react with, absorb, or even dissolve substances in contact with it. Thus, incompatibilities between elastomer closures and injectable solutions occur frequently, thereby presenting many problems. One such very important problem is the interaction between rubber closures and bacteriostatic agents present in the solutions. These agents are added to multiple-dose parenteral preparations to insure bacteriostasis for the life of the product. Loss of preservative from solution by interaction with rubber closures could result in bacterial contamination of the injectable preparation if bacteria were accidently introduced.

The 1953 edition of the British Pharmacopoeia (1) recognizes the tendency of rubber to absorb preservatives from injectable solutions. It therefore directs that closures for parenteral solutions should be boiled in several changes of distilled

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water and then either boiled under a reflux condenser for 30 minutes, or stored for not less than 48 hours in a solution containing the same bacteriostat in the same concentration as in the injectable solution, or preferably in twice this concentration. However, the British Pharmacopoeia adds the further precaution that in prolonged storage, rubber so treated is still likely to absorb preservative from the injectable solution.

As early as 1923, Masucci and Moffat (2) reported on the substantial loss of cresol or phenol from rubber-capped vials of solutions stored at room temperature. The loss of preservative was explained as due to the vaporization of cresol or phenol through the rubber caps. In 1937 McGuire and Falk (3), studying certain biological products, found that 0.5% phenol was reduced to 0.3% after 237 days at 37°, while controls with glass-stoppered containers underwent no diminution of preservative content.

Within recent years, the influence of vial stoppers on the preservative content of parenteral products has received considerable attention. Wiener (4) studied the effect of various composition rubber closures on the bacteriostatic action of thiomersolate and observed great variability as to their interference with antibacterial properties. Berry (5) has shown that rubber caps could reduce the strength of a 0.1% chlorocresol solution by as much as 75% under normal conditions of storage. Wing (6–8) and Nielsen (9) studied the absorption of phenol and chlorocresol by elastomer caps. Wing (8) also investigated the effects of varying the chemical composition of the rubber mix on the absorption of these preservatives. Royce and Sykes (10) determined the loss of phenol, cresol, benzyl alcohol, chlorocresol, chlorobutanol, and phenylmercuric nitrate from rubber-closed multiple-dose containers. Of these preservatives, phenol and benzyl alcohol were the least affected and phenylmercuric nitrate the most affected. These investigators also evaluated the methods recommended in the British Pharmacopoeia for the pretreatment of rubber closures and found that they were not adequate to equilibrate the rubber with the preservative in solution. Wiener (4) also found this to be true in the case of thiomersolate.

It is evident from these studies that a considerable amount of work has been done relating the effects of rubber closures on preservative agents. In most instances, however, the investigators did not define the compositions of the rubber closures used. It must therefore be realized, when considering these data, that another composition closure might not produce the same effects. Siddell (11) reviewed the problems as they relate to chemical uniformity of vial stoppers. He attributed the existence of these problems to inadequate quality standards and poor control of methods of manufacture. Since 1954, attempts have been made in the United States and England to develop standards which would control the composition and properties of rubber closures. The lack of success in this endeavor is evident in the U.S.P. XVI, whose only specification for rubber closures is that "containers and closures shall not physically or chemically interfere with the preparations in any manner to alter the strength, quality, or purity beyond the official requirements."

In most of the previous work, little consideration was given to the possible degradation of preservative in solution. Generally, only the absorption or diffusion characteristics were evaluated. This study evaluates several preservatives with three commonly used rubber closures for (*a*) preservative degradation in solution, (*b*) loss of preservative due to absorption or diffusion into and through the closure, (*c*) influence of chemical structure on diffusion characteristics, (*d*) temperature dependency of the degradative and diffusion processes, (*e*) relationship of preservative loss to microbiological activity, and (*f*) changes in the physical properties of the rubber in contact with the preservative solutions.

EXPERIMENTAL

Materials.—0.275 *M* Citric acid-sodium phosphate buffer of pH 4.0. *p*-Chloro-*β*-phenylethyl alcohol, Ciba, b.p. 80–83° at 1.07 mm. Phenylethyl alcohol, Eastman Organic Chemicals. Benzyl alcohol, reagent grade, Fisher Scientific Co. Methylparaben U.S.P. Chlorobutanol, anhydrous, U.S.P. Elastomer closures: V-32-natural crepe, neoprene polymer, and butyl polymer, West Co. U.S.P. type I, 10-ml. amber ampuls and vials, Kimble Glass Co. Three piece aluminum caps for vials, West Co., No. 13-30.

Equipment.—Beckman spectrophotometer model DU. Beckman spectrophotometer model DK-2. Shore type A hardness tester. Beckman pH meter model G.

Preparation of Closures and Vials. The stoppers used in this study were immersed in a 1.0% solution of tetrasodium pyrophosphate and heated at 90° for 15 minutes under constant agitation. They were removed from the solution and washed 10 times with distilled water and then five times with filtered water for injection. The stoppers were dried at 40° for 48 hours before use.

The ampuls and vials were washed, placed into special pans, and covered. These pans of ampuls and vials were sterilized at 180° for 2 hours.

Preparation of Ampuls and Vials of Preservative Solution.—Solutions containing (*a*) 0.3% *p*-chloro-*β*-phenylethyl alcohol, (*b*) 0.5% phenylethyl alcohol.

(*c*) 1.0% benzyl alcohol, (*d*) 0.2% methylparaben, and (*e*) 0.5% anhydrous chlorobutanol, on a weight to volume basis, were prepared with water for injection buffered to pH 4.0. The preservative solutions were then passed through medium porosity sintered-glass filters. Each preservative solution was filled into 10-ml. amber ampuls and vials. The ampuls were closed by customary pull sealing techniques under an oxygen-gas flame. The vials of each preservative were stoppered with three different composition closures. The stoppered vials were then sealed with three-piece aluminum caps at a constant sealing head pressure of 60 p.s.i. with a Westcapper. Samples of buffer solution in ampuls and vials were filled and sealed similarly. The buffer and preservative solutions in ampuls and vials were placed into constant temperature ovens regulated at 25, 40, 50, and 60, ±1.5°. Half of the vials were stored upright and half inverted. The constant temperature equipment employed has been described in a previous publication (12). At designated time intervals, samples were withdrawn and tested for residual preservative content, microbiological activity, pH, and physical changes. In addition, physical tests were performed on the closures to determine whether or not changes had taken place as to hardness, shape, and color. As a control, buffer solutions in ampuls and vials were run concurrently.

Loss of Preservative Upon Sterilization.—Samples of each of the five preservative solutions in vials stoppered with natural, neoprene, or butyl rubber and in ampuls were autoclaved at 115°, 10 p.s.i., for 30 minutes. Assay for residual preservative content was then performed.

Apparent Distribution of Preservative Between Buffer Solution and Rubber.—Ten-millimeter aliquots consisting of (*a*) *p*-chloro-β-phenylethyl alcohol, (*b*) phenylethyl alcohol, and (*c*) chlorobutanol were filled into 20-ml. amber ampuls. The ampuls containing each preservative were separated into three groups. In one group, 5 Gm. of natural rubber stoppers (cut in half) were placed into each ampul; into each ampul of the second group cut neoprene polymer stoppers were added. No stoppers were added to the ampuls of the third group. The ampuls were pull sealed and placed into constant temperature ovens regulated at 25 and 40 ±1.5°. At the end of 4 weeks' storage the ampuls were removed and the solution assayed for residual preservative. Apparent distribution coefficients were calculated as follows

$$K_T = \frac{10(C_A - C_B)}{C_B V} = \frac{C_R}{C_B}$$

where K_T = apparent distribution coefficient at temperature T, C_A = concentration of preservative in ampul solution after storage without closures (mg./ml.), C_B = concentration of preservative in ampul solution after storage with 5 Gm. of closures (mg./ml.), V = volume of 5 Gm. of rubber, $10(C_A - C_B)$ = concentration of preservative in rubber (mg.), and $[10(C_A - C_B)]/V = C_R$ = concentration of preservative in rubber calculated as mg./ml.

To avoid discrepancies in the distribution values due to degradation of preservative in solution, the concentration of preservative in the ampul solution without closures, after 4 weeks' storage at the above temperature conditions (C_A), was taken as the total preservative concentration for the calculation of K_T.

Analytical Methods. — *Chlorobutanol.* — The method employed is essentially that of Rehm and Mader (13) with minor changes. The undegraded chlorobutanol in solution was determined by pipetting a 3-ml. aliquot of solution into a suitable microsteam distillation apparatus. One drop of concentrated sulfuric acid was added and the sample steam distilled for 3 minutes. The condenser was washed down with warm distilled water and the rinsings added to the distillate to make a total volume of 50 ml. The remainder of the procedure was according to the method of Rehm and Mader in the absence of interfering substances: mg. chlorobutanol/ml. = A sample/A standard × 0.5 × dilution factor measured at the absorption maximum of 500 mμ.

p-Chloro-β-phenylethyl Alcohol.—Residual preservative was determined by pipetting 5 ml. of solution into a microsteam distillation apparatus. The sample was steam distilled and 40 ml. was collected. Then 50 ml. of methanol was added to the distillate and brought up to volume with distilled water in a 100-ml. volumetric flask. Absorbance was measured at 267.5 mμ where $A(1\%, 1\text{ cm.}) = 22$.

Phenylethyl Alcohol.—The concentration of preservative was determined by pipetting 3 ml. of solution into a microsteam distillation apparatus and the sample steam distilled to 30 ml. The distillate was brought up to 50 ml. in a volumetric flask with distilled water. Absorbance was measured at 257 mμ where the $A(1\%, 1\text{ cm.}) = 18$.

Benzyl Alcohol.—The method employed was similar to that used for phenylethyl alcohol, except that 1 ml. of solution was pipetted into a microsteam distillation apparatus and steam distilled to 25 ml. and absorbance was measured at 257 mμ where $A(1\%, 1\text{ cm.}) = 18$.

Methylparaben.—A 5-ml. aliquot was pipetted into a 100-ml. volumetric flask and brought up to volume with methanol. Five milliliters of this solution was transferred to another 100-ml. volumetric flask and brought up to volume with methanol. Absorbance was measured at 256 mμ where $A(1\%, 1\text{cm.}) = 1{,}115$.

Zinc Mercaptobenzothiazole.—This substance was leached from the rubber and detected by the presence of a sharp peak at 318 mμ in the ultraviolet spectra of the preservative solutions. The concentration of this material was measured at 318 mμ where the $A(1\%, 1\text{ cm.}) = 16.7$.

Chromatography of Degraded Samples.—Thin-layer chromatography, according to the method of Stahl (14), was performed on each of the preservatives after storage in vial solutions at a pH of 4.0 and at 60° for 12 weeks. The concentrations of preservative placed on the layered plates were (*a*) 60 γ *p*-chloro-β-phenylethyl alcohol, (*b*) 100 γ phenylethyl alcohol, (*c*) 200 γ benzyl alcohol, (*d*) 40 γ methylparaben, and (*e*) 100 γ chlorobutanol. Silica gel G was used as the layer and the solvent system employed was benzene-acetone (1:1). The detecting reagent used for spraying the plates was composed of equal volumes of 1% aqueous solutions of ferric chloride and potassium ferrocyanide.

Measurement of Rubber Hardness.—A Shore type A-2 hardness tester was used. Measurements were made on the flange of the stoppers and readings were taken in triplicate and averaged.

Microbiological Tests.—*Bactericidal Activity of Preservatives.*—The phenol coefficient-type test employed involved the contamination of various concentrations (in pH 4 buffer) of the five preservatives under study with *S. aureus* and *E. coli*. The concentration of preservative necessary to destroy all the test organisms in less than 15 minutes, but not in 10 minutes, was determined by subculturing into fresh broth after contact periods of 5, 10, and 15 minutes.

Self-Sterilizing Properties of the Preservatives.—Solutions of the five preservatives, buffered to pH 4 and at the concentration noted earlier in this section, were evaluated as to the time required to destroy 50 and 95% of the following test organisms: *E. coli*, *S. aureus*, and the spores of *B. cereus* var. *mycoides*. Plate counts for the number of surviving organisms were performed after 1, 3, 5, 24, 48, 72, and 96 hours, and again after 7 days. In addition, at these time intervals, aliquots were tested for sterility by transferring to thioglycollate fluid medium.

Correlation of Chemical Assay to Microbiological Activity.—The microbiological assay method was investigated with only *p*-chloro-β-phenylethyl alcohol. It involved the development of a standard curve by using preservative concentrations in pH 4 buffer solution from 0.3 to 0.06% and diluting 1:7 with buffer solution before contamination. The solutions were contaminated by adding 1-ml. amounts of an 18-hour diluted culture (91% light transmission in a Bausch and Lomb Spectronic 20 colorimeter fitted with a No. 580 light filter) of *E. coli* to 14-ml. quantities of solution. At various time intervals, 1-ml. portions were removed and plated in trypticase soy agar for survivor count. Curves were plotted of per cent survivors *vs.* time for each concentration and the time required to produce 50% killing was obtained from the plots. A standard curve was then drawn of the time required to kill 50% of the organisms *vs.* concentration of preservative. Using the 50% killing time value of a degraded sample of preservative and placing it on the standard curve, an estimate of the residual concentration of preservative was obtained.

RESULTS AND DISCUSSION

The decrease in concentration of several bacteriological preservatives in aqueous buffered solutions stored in rubber-stoppered vials was investigated. These solutions were buffered to a pH of 4 in order to maintain a constant hydrogen ion concentration as well as to permit the study of chlorobutanol at a pH in which degradation in solution would be minimal.

The stoppers chosen for evaluation are representative of three rubber compositions commonly employed for multiple-dose vial closures. The composition and per cent rubber content of these stoppers are presented in Table I. Several of the physical properties of these closures are given in Table II.

Chemical and Physical Results.—An ascending thin-layer chromatographic technique was employed to distinguish decreases in preservative content in rubber-stoppered vial solutions which had been stored at 60° for 12 weeks. From the chromatogram in Fig. 1, it was estimated that in the case of chlorobutanol approximately a 90% reduction in concentration had occurred while for the other preservatives about a 50% reduction in concentration resulted. Consequently, additional studies were performed to ascertain the portion of preservative lost due to degradation in solution as well as the amount lost by reaction with the rubber stopper.

TABLE I.—CLOSURE COMPOSITION

Natural Rubber	Neoprene Rubber	Butyl Rubber
Natural crepe[a]	Neoprene polymer[b]	Butyl polymer[c]
Titanium dioxide	Sulfonated oil	Barium sulfate
Barium sulfate	Calcined clay	Calcined clay
Zinc oxide	Barium sulfate	Carbon black
Iron oxide	Zinc oxide	Titanium dioxide
Diphenylamine-acetone condensation product	Magnesium oxide	Stearic acid
	Iron oxide	Paraffin wax
Thiuram combined with aniline reaction product	Stearic acid	Sulfur
	Mineral oil	Thiazole-dithiocarbamate accelerator
	Imidazoline type accelerator	

[a] Rubber content, 50.4% of total composition. [b] Rubber content, 42.4% of total composition. [c] Rubber content, 36.9% of total composition.

TABLE II.—PHYSICAL CHARACTERISTICS OF THE RUBBER STOPPERS

Properties	Stopper Composition		
	Natural Rubber	Neoprene Rubber	Butyl Polymer
Specific gravity	1.600	1.490	1.609
Thickness[a]	0.125 inch	0.125 inch	0.124 inch
Weight[b]	618 mg.	548 mg.	595 mg.

[a] Measured microscopically and value is average of five measurements of different stoppers. [b] Value is average of 10 stoppers.

Chlorobutanol.—Chlorobutanol has been used widely as a bacteriostatic agent in parenteral solutions. The results of a number of studies on the stability of chlorobutanol in solution have been reported (15–20). The majority of these reports (15, 17–20) are concerned with the instability of chlorobutanol solutions during autoclaving. Several investigators (18, 19) have found that decomposition is minimal at pH 5 or below. Recently, a comprehensive quantitative study of the degradation of chlorobutanol was performed by Nair and Lach (21) who evaluated the kinetics of the degradation over a pH range from 2 to 7.5. From pH 2 to 4 the decomposition was found to be pH independent and having a half-life of about 90 years at 25°. However, at pH 7.5, the half-life dropped sharply to slightly less than 3 months. Therefore, for pharmaceuticals containing chlorobutanol as the preservative, it appears desirable to buffer the solution to pH 4 or below in order to minimize the degradation reaction.

In this investigation, the influence of natural, neoprene, and butyl rubber stoppers on the concentration of chlorobutanol in vial solutions buffered at pH 4 was studied. The residual concentrations of chlorobutanol in ampul solutions found at different time intervals and at several elevated temperatures are shown in Fig. 2. It is evident from the plot that the degradation reaction is first order with respect to chlorobutanol concentration. Using the integrated Arrhenius equation, the heat of activation was calculated employing the rate constants at

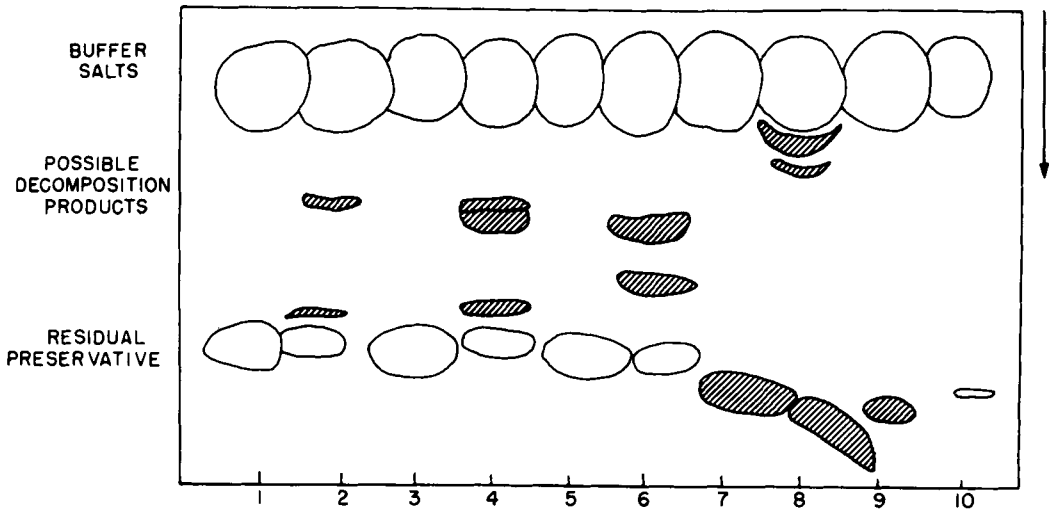


Fig. 1.—Thin-layer chromatograms of aged (60° for 12 weeks) and freshly prepared solutions of the five preservatives. 1, *p*-Chloro-β-phenylethyl alcohol standard solution; 2, *p*-chloro-β-phenylethyl alcohol after storage; 3, phenylethyl alcohol standard solution; 4, phenylethyl alcohol after storage; 5, benzyl alcohol standard solution; 6, benzyl alcohol after storage; 7, methylparaben standard solution; 8, methylparaben after storage; 9, chlorobutanol standard solution; 10, chlorobutanol after storage. ▨, Blue stain; □, white stain.

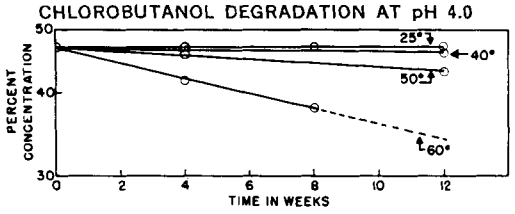


Fig. 2.—Plot of the logarithm of the per cent residual concentration of chlorobutanol against time at pH 4.0.

50 and 60°. A value of 27.6 Kcal. was obtained as the heat of activation for the degradation of chlorobutanol at pH 4. This is a reasonable value for a hydrolytic reaction of this nature and is in fair agreement with the value of 30.7 Kcal. obtained by Nair and Lach (21) for this reaction.

A comparison of chlorobutanol loss in vials stoppered with these different composition closures with that found for solutions in ampuls is presented in Table III. It is evident from these data that the closures have a marked deleterious effect on preservative concentration at all temperatures. This greater loss of preservative in the vial solutions can be attributed to (*a*) adsorption and absorption of preservative by the rubber, (*b*) possible diffusion of the preservative through the rubber and subsequent volatilization into the atmosphere, and (*c*) interaction of preservative with material extracted from the closure. Of these three factors, adsorption and absorption of the preservative by the closure is probably responsible for the greatest loss.

In Table IV are presented data indicating the per cent of chlorobutanol lost from solution due to the influence of the rubber stopper. These values were obtained by subtracting the per cent residual preservative found in the vial solutions from that found in the ampul solutions under the same storage conditions. From the data in this table and that in Table III, it is demonstrated that the major quantity of preservative lost due to rubber stopper effect takes place during the first 2 weeks of storage. Thereafter, very little if any, further loss occurs. This may be ascribed to the development of an apparent equilibrium state between the concentration in the solution and in the stopper.

It is interesting to note that the storage of vial solutions in an inverted position seems, for the most part, to cause a greater loss of preservative from solution than occurs in those vials stored in an upright position. Since it is not uncommon for vials to be on their side during storage in pharmacies, hospitals, or physicians' offices and thereby permitting the contents to come in contact with the rubber stopper, the losses of preservative in vials stored in the inverted position warrant serious consideration.

According to the 60° storage data in Table IV, it is evident that the preservative loss due to the rubber stopper decreases with the increase of preservative degradation as indicated by the ampul assays in Table III. This effect can be explained by the significant degradation of preservative in solution which, in turn, results in a reduction of the amount of available chlorobutanol to equilibrate between the solution and closure.

The data in Tables III and IV indicate that the neoprene rubber stopper is responsible for the greatest loss of chlorobutanol from solution. At 60°, after 12 weeks' storage, the vials in the inverted position show only an 8.5% residual concentration, whereas the ampuls under the same storage conditions contain 72.4%. These results are in agreement with the estimate of 10% residual preservative obtained from the thin-layer chromatogram. The other two stoppers, natural and butyl rubber, exert approximately the same effect on preservative loss with possibly the natural rubber stopper exerting a slightly lesser deleterious effect. This substantially

TABLE III.—INFLUENCE OF DIFFERENT COMPOSITION CLOSURES ON THE PER CENT RESIDUAL CONCENTRATION OF CHLOROBUTANOL IN VIAL SOLUTIONS AFTER STORAGE

Temp., °C.	Storage Time, wk.	Ampul Control	Stopper Composition: Natural Rubber Upright	Natural Rubber Inverted	Neoprene Polymer Upright	Neoprene Polymer Inverted	Butyl Polymer Upright	Butyl Polymer Inverted
25	2							
	8	100	87.3	83.0			74.5	63.8
	12	100	81.0	78.7	87.3	81.0	68.1	63.8
40	2	100	76.6	63.8	61.7	59.6	68.1	61.7
	8	100	76.6	63.8	57.5	51.0	68.1	61.7
	12	97.9	74.5	57.5	53.2	46.8	66.0	66.0
50	2	97.9	59.6	57.5	57.5	46.8	66.0	61.7
	8	93.6	57.5	....	59.6	46.8	66.0	59.6
	12	91.5	57.5	42.5	46.8	38.3	59.6	57.5
60	2	95.8	57.5	53.2	51.0	46.8	51.0	48.9
	8	81.0	59.6	51.0	53.2	42.5	51.0	48.9
	12	72.4	57.5	42.5	29.8	8.5	46.8	46.8

TABLE IV.—PER CENT CHLOROBUTANOL LOSS DUE TO STOPPER

Temp., °C.	Storage Time, wk.	Natural Rubber Upright	Natural Rubber Inverted	Neoprene Rubber Upright	Neoprene Rubber Inverted	Butyl Rubber Upright	Butyl Rubber Inverted
25	2						
	8	12.8	17.0			25.6	36.2
	12	19.1	21.3	12.8	19.1	31.9	36.2
40	2	23.4	36.2	38.4	40.0	31.9	38.4
	8	23.4	36.2	42.6	49.0	31.9	38.4
	12	23.4	40.0	44.7	51.1	31.9	31.9
50	2	38.4	40.0	40.0	51.1	31.9	36.2
	8	36.2	....	34.0	46.8	27.7	34.0
	12	34.0	49.0	44.7	53.2	31.9	34.0
60	2	38.4	42.6	44.7	49.0	44.7	46.8
	8	21.3	29.8	27.7	38.4	29.8	31.9
	12	14.9	29.8	42.6	63.8	25.6	25.6

greater effect caused by neoprene stoppers can essentially be explained by the physical properties of this rubber as compared with the other two. It is apparent from the data in Table II that neoprene rubber is of a more porous composition than either natural or butyl rubber. As a result of this greater porosity, chlorobutanol could diffuse into the closure with less impediment. The physical properties listed in Table II for the natural and butyl rubber stoppers indicate that the porosity of these two rubbers are approximately the same. Accordingly, if the chlorobutanol loss from solution is due essentially to absorption by the rubber, then the quantity absorbed should be approximately the same for both elastomers which, according to the data in Tables III and IV, proved to be the case.

Of particular interest are the results pertaining to the loss in preservative content from vial solutions at room temperature. Although the ampuls stored for 12 weeks at 25° show no loss of chlorobutanol content, the vial solutions stored in an inverted position undergo a loss ranging from 19 to 36%, depending upon the stopper used. These results are not surprising in the light of the report by Royce and Sykes (22) which indicated that a 0.5% aqueous solution of chlorobutanol was partitioned between rubber and water in the ratio of 85 to 15% after one month storage at room temperature. In their partition studies, these investigators used a ratio of 1 Gm. rubber to 3 ml. of solution.

During the study with chlorobutanol solution, it was found that zinc mercaptobenzothiazole, an accelerator used as part of the curing system in the butyl rubber stopper, was extracted by the solution, forming a fine colloidal precipitate in the vial solution. In addition, the portion of the closure in contact with the preservative solution became discolored and the solution developed a yellow color. Preliminary tests with other preservatives in solution showed similar results. As a result of the physical incompatibility of this rubber stopper with the various preservative solutions, it was eliminated from comprehensive evaluation with the other preservatives.

p-Chloro-β-phenylethyl Alcohol.—Hess and Speiser (23, 24) in their studies on the comparative efficiency of bactericidal compounds in buffer solutions, showed that *p*-chloro-β-phenylethyl alcohol exhibited promising potentialities as a new bactericidal agent. Studies performed in our laboratories have confirmed their findings, and additional data are presented in a later section of this paper.

In order to evaluate the stability of *p*-chloro-β-phenylethyl alcohol in vials stoppered with natural and neoprene rubber stoppers, a 0.3% solution buffered at pH 4 was employed. As controls, ampul solutions were used at each storage condition. According to the data given in Table V, the closures have a significant deleterious effect on preservative concentration at the several storage conditions. As in the case of the chlorobutanol solution, the neoprene closure causes a significantly greater loss of preservative content than does the natural rubber stopper.

The per cent *p*-chloro-β-phenylethyl alcohol lost due to the closure is summarized in Table VI. These values are representative of the per cent residual preservative in the vial subtracted from the

TABLE V.—INFLUENCE OF DIFFERENT COMPOSITION CLOSURES ON THE PER CENT RESIDUAL CONCENTRATION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL IN VIAL SOLUTIONS AFTER STORAGE

Temp., °C.	Storage Time, wk.	Stopper Composition: Natural Rubber — Ampul Control	Natural Rubber — Upright	Natural Rubber — Inverted	Neoprene Rubber — Ampul Control	Neoprene Rubber — Upright	Neoprene Rubber — Inverted
25	2						
	8	100	98.6	82.8	100	82.8	69.0
	12	100	82.8	76.0	100	76.0	65.5
40	2	100	98.6	86.2	100	86.2	72.5
	8	100	79.3	76.0	100	72.5	62.0
	12	89.6	76.0	72.5	89.6	62.0	51.7
50	2	100	86.2	82.8	100	79.3	65.5
	8	96.6	76.0	72.5	96.6	62.0	55.2
	12	89.6	72.5	69.0	89.6	55.2	44.8
60	2	96.6	82.8	79.3	96.6	72.5	62.0
	8	86.2	69.0	65.5	86.2	62.0	48.3
	12	86.2	65.5	58.6	86.2	48.3	41.1

TABLE VI.—PER CENT *p*-CHLORO-β-PHENYLETHYL ALCOHOL LOSS DUE TO STOPPER

Temp., °C.	Storage Time, wk.	Natural Rubber — Upright	Natural Rubber — Inverted	Neoprene Rubber — Upright	Neoprene Rubber — Inverted
25	2				
	8	10.4	17.2	17.2	31.0
	12	17.2	24.0	24.0	34.5
40	2	10.4	13.8	13.8	27.5
	8	20.7	24.0	27.5	38.0
	12	13.6	17.1	27.6	37.9
50	2	13.2	17.2	20.7	34.5
	8	20.6	24.1	34.6	41.4
	12	17.1	20.6	34.4	44.8
60	2	13.8	17.3	24.1	34.6
	8	17.2	20.7	24.2	37.9
	12	20.7	27.6	37.9	44.8

per cent remaining in the ampuls at the particular condition and time of storage. Again, as in the chlorobutanol solution study, the largest loss of preservative content takes place during the first 2 weeks of storage, with subsequent loss being relatively small. However, the loss of preservative due to closure effect is substantially less in the case of this preservative as compared to that for chlorobutanol. This may be explained by the apparent distribution coefficient of the preservatives between solution and rubber, where chlorobutanol favors the rubber to a greater extent than does *p*-chloro-β-phenylethyl alcohol. This is discussed in greater detail in a subsequent section of this report.

It is interesting to note (Table VI) that for the vials stoppered with the neoprene rubber and stored in an inverted position, there is a substantially greater loss in preservative content than occurs in the vials stored upright. However, for vials stoppered with natural closures, the loss of preservative from solution in the inverted vials does not differ significantly from that lost in the upright vials.

Of major importance is the loss of preservative content which takes place when the vial solutions are stored at room temperature for 12 weeks. It can be seen from Table VI that vials stored in an inverted position lose between 24 to 34% preservative from solution, the larger quantity being from the vials stoppered with neoprene closures. Because of this substantial loss in preservative content, it would seem that this preservative as well as chlorobutanol should not be used in vial solutions unless prior closure-preservative studies are performed. The ampul solutions used as controls showed no loss in preservative content during the storage period.

Phenylethyl Alcohol, Benzyl Alcohol, and Methylparaben.—Preliminary studies of the type just described for chlorobutanol and *p*-chloro-β-phenylethyl alcohol have been performed for phenylethyl alcohol, benzyl alcohol, and methylparaben. The results obtained indicate that these preservatives are also absorbed by the closures. Comprehensive studies of these preservatives are under way and the results will be reported at a later date.

Influence of Autoclaving Conditions on Preservative Loss.—Vial solutions of the several preservatives buffered at pH 4 and stoppered with neoprene, natural, and butyl rubber closures were autoclaved at 115°, 10 p.s.i. for 30 minutes. The residual preservative content in these solutions was determined and the data are summarized in Table VII. It is apparent from these results that the vial solutions containing *p*-chloro-β-phenylethyl alcohol are most deleteriously affected with respect to residual preservative concentration. Of the closures tested, natural rubber is the least reactive. It should be noted that the only ampul solution undergoing loss in preservative content after autoclaving was chlorobutanol. A 5% loss in preservative content took place in the ampul solutions of chlorobutanol. Nair and Lach (21), in their kinetic studies of chlorobutanol degradation in solution, found that at pH 5 there occurred a 13% loss of chlorobutanol content upon autoclaving at 115°, 10 p.s.i. for 30 minutes.

TABLE VII.—EFFECT OF AUTOCLAVING AT 115°, 10 p.s.i. FOR 30 MINUTES ON THE PRESERVATIVE CONTENT IN VIAL SOLUTIONS STOPPERED WITH DIFFERENT COMPOSITION RUBBER CLOSURES

Preservative[a]	% Residual Preservative — Ampul Control	Natural Rubber	Neoprene Rubber	Butyl Rubber
p-Chloro-β-phenylethyl alcohol	100	91	88	88
Phenylethyl alcohol	100	100	96	98
Chlorobutanol	95	92	92	92
Benzyl alcohol	100	100	90	100
Methylparaben	100	100	100	100

[a] Preservative solutions were buffered to pH 4.0.

Since the half-life of the degradation reaction at pH 4 is about 2.2 times that at pH 5, the value of 5% degradation obtained in this study seems to be entirely in order.

Apparent Distribution of Preservative Between Rubber and Solution.—It has been reported by Wing (7) and Royce and Sykes (22) that the proportion of chlorocresol absorbed by rubber is much higher than that of phenol. This results in partition coefficients which are almost 20 times greater than for phenol with the same rubber. It would appear that the chlorine substituent on the cresol molecule greatly influences the diffusion of the preservative into the rubber.

In order to determine whether a similar situation existed for *p*-chloro-β-phenylethyl alcohol and phenylethyl alcohol, distribution studies were performed. The results obtained are summarized in Table VIII. These data indicate that the chlorinated compound distributes more strongly into the rubber than does the nonchlorinated analog. In addition, the neoprene rubber absorbs to a greater degree than does the natural rubber. The data show that the distribution values decrease with a rise in temperature, with the exception of neoprene rubber in contact with *p*-chloro-β-phenylethyl alcohol solution. In this case the 40° distribution value is greater than for the 25° value.

TABLE VIII.—APPARENT DISTRIBUTION OF PRESERVATIVES BETWEEN RUBBER AND BUFFER SOLUTION[a] AFTER 4 WEEKS STORAGE

$K_T = C_R/C_B$

Temperature, °C.	Phenylethyl Alcohol[b]		*p*-Chloro-β-phenylethyl alcohol[c]	
	Natural	Neoprene	Natural	Neoprene
25	1.72	4.23	6.05	16.4
40	1.39	4.13	5.70	21.8

[a] Solutions buffered to a pH of 4.0. [b] Ampul concentration at end of 4 weeks: 25° = 0.46%, 40° = 0.43%. [c] Ampul concentration at end of 4 weeks: 25° = 0.26%, 40° = 0.25%.

Of the closures investigated, the butyl polymer was the only one to exhibit significant physical incompatibilities. With each of the preservative solutions, zinc mercaptobenzothiazole was leached from the closure, forming a colloidal precipitate in the vial solutions. That portion of the closure in contact with the solution became discolored and the solutions developed a yellow color. Hardness testing of the three closures in contact with the preservative solutions at different temperature conditions showed no significant changes when compared to original values.

Results of Microbiological Tests.—In order to evaluate the comparative bactericidal activities of the five preservative solutions under study, a phenol coefficient type test was performed with *S. aureus* and *E. Coli.* The data obtained from this study are summarized in Table IX. It is evident from these results that *p*-chloro-β-phenylethyl alcohol is the most active of these preservatives. In comparison to its nonhalogenated analog, phenylethyl alcohol, it is approximately five times more active when tested with *S. aureus* and approximately three times more active when the test organism is *E. coli.* This superior activity of the halogenated molecule may be explained according to the theory of Hess and Speiser (24) who have postulated the existence of a lipid barrier at the cell surface of the organisms. Since the halogenated molecules distribute more readily into nonpolar solvents, it would be expected to penetrate the lipid barrier more rapidly than the nonhalogenated molecule and, hence, require a smaller concentration to achieve the same degree of activity. It is probably this same property which causes chlorinated preservatives to be absorbed by rubber closures to a greater extent than the nonhalogenated analogs.

TABLE IX.—RELATIVE BACTERICIDAL ACTIVITY OF PRESERVATIVES

Preservative[a]	Lowest Concentration, %, Required to Destroy All Test Organisms in 15 Minutes or Less, But Not in 10 Minutes	
	S. aureus	*E. coli*
p-Chloro-β-phenylethyl alcohol	0.10	0.15
Chlorobutanol	0.28	0.30
Phenylethyl alcohol	0.50	0.50
Benzyl alcohol	0.90	0.80
Methylparaben	0.25	0.25

[a] All preservative solutions were buffered to pH 4.0.

Studies of the self-sterilizing properties of the five preservatives were carried out at concentrations most commonly used for parenteral solutions. The results of this study are given in Table X. These data show that all the preservatives tested are very effective against *S aureus* and *E. coli.* However, against spores of *B. cereus*, benzyl alcohol seems to be the most effective preservative, with *p*-chloro-β-phenylethyl alcohol next in effectiveness. Of the five preservatives, phenylethyl alcohol is the least effective as a sporicidal agent.

Assays based on the chemical analysis of preservative in pharmaceutical solutions may, in certain instances, yield data of questionable significance. It is conceivable that a chemical breakdown of an effective preservative, as for example the loss of a chlorine substituent, may yield a product which *per se* possesses intrinsic antibacterial properties. Thus, an assay which is indicative of chemical breakdown or alteration of a preservative can be misleading, since the degraded product may exert demonstrable antimicrobial activity.

In view of these theoretical considerations, it was of interest to attempt the correlation of a chemical breakdown of *p*-chloro-β-phenylethyl alcohol in solution with a loss, if such should prove to be the case, of antibacterial activity as determined by microbiological methods. The method used for determining the latter activity involved the establishment of a standard curve obtained by plotting the ET_{50} (time required for freshly prepared standard solutions to destroy 50% of the test organisms) *vs.* the logarithm of preservative concentration. The best fitting straight line was computed by the method of least squares. Because of difficulties experienced thus far in reproducing the slope of the standard curve, the microbiological data with respect to the assay of chemically degraded solutions are not available at this time for interpretation. The results of this study, however, will be presented at a later date.

TABLE X.—SELF-STERILIZING PROPERTIES OF SELECTED PRESERVATIVES

Preservative	Time Required to Destroy the Following Percentages of Test Organisms,[a] hr. 50%			95%		
	E. coli.	*S. aureus*	Spores of *B. cereus*	*E. coli*	*S. aureus*	Spores of *B. cereus*
pH 4 Buffer solution	1–3	1–3	168	5–24	3–5	>168
p-Chloro-β-phenylethyl alcohol (0.3%)	<1	<1	5–24	<1	<1	48–72
Phenylethyl alcohol (0.5%)	<1	<1	48	<1	<1	<168
Benzyl alcohol (1.0%)	<1	<1	5	<1	<1	48–72
Methylparaben (0.2%)	<1	<1	24–48	<1	<1	<168
Chlorobutanol (0.5%)	<1	<1	24–48	<1	<1	>168

[a] Inoculum: *E. coli*, 700,000; *S. aureus*, 500,000; *B. cereus* spores, 50,000.

It is evident from this investigation that the choice of a rubber stopper for a particular vial solution requires considerable study to select one which exhibits optimal stability. Such a study should include the testing of the vial solutions stored in an upright as well as in an inverted position. In order to accomplish this most effectively, close cooperation must exist between the pharmaceutical and rubber stopper manufacturers.

Additional studies are under way to determine the effect of coated stoppers on preservative loss from solution and the influence of nonaqueous parenteral vehicles on closure properties.

SUMMARY

In this investigation an attempt was made to determine the contributing factors responsible for the loss of preservative from multiple-dose vial solutions. The preservatives evaluated were (*a*) chlorobutanol, (*b*) *p*-chloro-β-phenylethyl alcohol, (*c*) phenylethyl alcohol, (*d*) methylparaben, and (*e*) benzyl alcohol. Solutions of these preservatives in multiple-dose vials were stoppered with three commonly used rubber closures representing natural rubber, neoprene polymer, and butyl polymer. The results obtained are summarized as follows:

1. Preservative solutions in vials stoppered with rubber closures exhibited a substantially greater loss in preservative content than ampul solutions of the same preservative.

2. Vials stored in an inverted position demonstrated a greater loss in preservative content than those stored upright.

3. The natural rubber stoppers exerted the least deleterious effect on preservative content in solution.

4. The butyl rubber closure was found to be physically incompatible with the preservative solutions tested due to a leaching of an accelerator residue.

5. Vial solutions of chlorobutanol and *p*-chloro-β-phenylethyl alcohol stored in an inverted position at room temperature exhibited as much as 30% reduction in preservative content in less than 8 weeks. Since it is not unusual for vials to be stored in a manner whereby their contents are in direct contact with the rubber stopper, these results warrant serious consideration when formulating multiple-dose vial preparations.

6. Distribution studies on *p*-chloro-β-phenylethyl alcohol and phenylethyl alcohol have shown that the chlorinated compound is absorbed into the rubber to a substantially greater degree than the nonchlorinated analog.

7. Results obtained from studies on the comparative bactericidal activity of the preservative solutions have shown that *p*-chloro-β-phenylethyl alcohol was the most effective agent aginst *E. coli* and *S. aureus*.

8. An attempt was made to relate loss in preservative content with corresponding changes in antimicrobial activity.

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Chick Embryo Technique for the Evaluation of Absorption of Radioactive Sodium Iodide from Ointment Bases

By JOHN S. RUGGIERO and DONALD M. SKAUEN

In view of the lack of agreement pertaining to the absorption of ingredients from ointment bases, an investigation was conducted to develop a procedure which would be suitable for this type of evaluation. A new method was developed which measures the percentage of absorption of radioactive sodium iodide by 11-day-old chick embryos. Penetration of the isotope was measured from the ointment base through the shell membrane and chorioallantoic membrane of the chick embryo to their thyroid glands.

EVER SINCE man first used a vehicle as a diluent for applying medication, a search has been conducted to find the "ideal ointment base" which could be used to give maximum release and penetration of the active ingredients. Unfortunately, much of the information that has been gathered in this field has led to confusion with many contradictory statements appearing in the literature. For example, Macht (1) states that lanolin and lard are unsatisfactory for absorption through the skin while Fayaud (2) claims that lanolin is best adapted for use as a base to be absorbed through the skin. According to Beeler (3), washable ointment bases permit the best penetration of medicaments, while Busse (4) claims that absorption through the skin depends exclusively on the nature of the substance which is incorporated and that the base makes little difference in the total absorption.

During the past half century many ingenious methods have been proposed to evaluate ointment bases according to their ability to release incorporated ingredients. In general, these methods may be classified either as *in vitro* or *in vivo* procedures. The *in vitro* methods (5–20) can contribute to our knowledge of the release of ingredients under certain specific conditions, but cannot be correlated with *in vivo* efficacy. On the other hand, although *in vivo* methods (21–36) are more applicable, they are usually limited by the unavailability of large numbers of experimental animals which are frequently needed to yield meaningful results.

Many of the aforementioned techniques have been superseded by radioisotopic procedures (37–48). These techniques offer a number of distinct advantages, especially since radioisotopes behave the same as their stable counterparts in chemical and biological processes. Also methods for their detection are so very sensitive, making it possible to test for minute quantities of the isotope.

Perhaps the major difficulty in attempting to develop a procedure for evaluating absorption lies in the fact that no animal possesses skin quite like that of man and, therefore, any correlations which have been made from experiments conducted *in vitro* or *in vivo* are necessarily limited. True, animal and human skin have many and important differences, however, experience indicates certain fields in which animal skin may be used to predict human skin reactions. Neuhauser (49), in seeking a form of keratin which would support the growth of dermatophytes, found a very good correlation between the physical similarities of human epidermis and the shell membrane of the avian egg. Hunter and Smith (50) have previously developed a technique that utilizes chick embryos for evaluating the release of antibiotics from various ointment bases through the shell membrane and chorioallantoic membrane. According to such a procedure, absorption would only be evaluated via the transepidermal route (51) since there are no appendages present to provide for transfollicular absorption. It is interesting to note, however, that even when absorption takes place through hair follicles and other appendages found in hairy animals, the absorbed ingredients must pass through at least one layer of epidermis, which is somewhat analogous to the thin tissue system of the chick embryo.

Radioactive isotopes were incorporated into ointment bases and chick embryos were employed as test animals in an attempt to develop a new technique for evaluating the efficiency of a base to release its active ingredients.

EXPERIMENTAL

The procedure that was developed utilizes radioactive sodium iodide (I^{131}), measuring the penetration of the isotope through the shell membrane and

Received June 17, 1960, from the Research Laboratories, University of Connecticut, School of Pharmacy, Storrs.
Accepted for publication June 16, 1961.
Presented to the Scientific Section, A.PH.A., Los Angeles meeting, April 1958.

chorioallantoic membrane to the thyroid gland of chick embryos (Fig. 1).

Following preliminary experiments indicating the degree of absorption of radioactive sodium iodide by chick embryos of various ages, it was decided that 11-day-old embryos which had been incubated with the isotope for 48 hours would be most suitable for use in evaluating the release of ingredients.

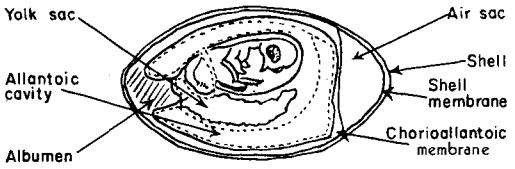


Fig. 1.—Schematic drawing of a chick embryo.

Preparation of the Ointment.—The ointment bases which were to be evaluated were prepared by carefully pipetting a calculated amount of radioisotope onto a known weight of ointment base that had been smeared onto a watch glass. After allowing this mixture to dry, the ointment was thoroughly mixed to insure uniform dispersion of the isotope.

Application of the Ointment.—Small glass rings were utilized in the application and standardization of the ointment in order to assure the fact that the ointment would come into contact with the same area of shell membrane in each embryo employed. These rings were cut from 10-mm. Pyrex tubing by a rotating carborundum wheel. The height of the rings was established by means of an adjustable stop which permitted little variation in height.

The rings were filled with ointment in much the same manner as one would fill an ointment jar. The rings were placed upon a piece of parchment paper and held firmly with a forcep while the ointment was added by means of a microspatula. It was possible to transfer the filled rings to the shell membrane of the embryos by first gently sliding the tip of a microspatula underneath the ring and then carefully pushing the ring from the spatula with the aid of a pair of forceps. Due to the delicate nature of the shell membrane, extra caution was employed when depositing the ring on the membrane so that trauma would not result and influence the degree of absorption.

The ointment was applied to the shell membrane by lowering the ring through a suitable opening which was made by piercing the shell at the air sac at a point where the shell membrane conveniently divides into two layers (Fig. 1). After application of the ointment, the opening in the air sac was sealed with Scotch Tape and the embryos were returned to an incubator at 37.5° where they were allowed to remain for 48 hours.

Determination of the Amount of Radioactivity Applied.—Usually three rings containing the ointment (more could be used depending on the total number of cases involved) were set aside from each batch of ointment used in order to ascertain the relative amount of radioactivity that was applied in each case. The amount of radioactivity in the rings, selected at random, was determined by dissolving the ointment in a suitable solvent, and then assaying various aliquots of the solutions. The result of averaging the radioactivity indicated in each of the samples was chosen to represent the relative amount of radioactivity applied in each case.

Determination of Amount of Radioactivity Absorbed by the Thyroid Glands.—After the radioactive ointment had remained in contact with the shell membrane for 48 hours, the eggs were removed from the incubator. The embryo was separated from the egg, taking care not to contaminate the embryo with any excess ointment that remained. The thyroid glands were viewed through a stereoscopic Bausch and Lomb microscope and removed with the aid of watchmaker's forceps. The glands were macerated with 10% sodium hydroxide solution, dried, and assayed for radioactivity.

Calculation of the Percentage of Absorption.—The percentage of radioactivity that was absorbed in each case was calculated by dividing the amount of radioactivity found in each thyroid by the amount of radioactivity that was applied. The percentage of absorption indicated by each of the embryos in the group was then averaged and reported as the percentage of absorption of radioactivity for a particular ointment base. A sample form showing all of the pertinent data is illustrated in Table I.

TABLE I.—EXAMPLE OF STANDARD FORM AND RESULTS OBTAINED FOR A TYPICAL OINTMENT BASE[a]

Embryo No.	C.p.m. in Thyroid above Background	Percentage of Applied Radioactivity in Thyroid
1	50.0	0.33
2	16.8	0.12
3	35.6	0.23
4	23.2	0.15
5	18.8	0.12
6	37.4	0.24
7	22.6	0.15
8	27.4	0.18
9	31.2	0.20
10	10.0	0.07
11	33.8	0.22
12	29.2	0.19
13	14.0	0.09
	0.176 ± 0.075 (mean % absorbed)	

[a] Amount applied, average of three standard rings, 15,360 c.p.m.; duration, 48 hours; base, Almay emulsion base (product of Almay, Inc.); background count, 22 c.p.m.

SUMMARY

A method is presented for determining the efficiency of ointment bases to release sodium iodide. The method utilizes the thin tissue system provided by the shell membrane and chorioallantoic membrane of 11-day-old chick embryos and the ability of the thyroid gland to absorb and concentrate radioactive sodium iodide. Such a system provides a rather simple, inexpensive, and rapid analysis of absorption.

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Evaluation of the Absorption of Radioactive Sodium Iodide from Various Ointment Bases by Means of a Chick Embryo Technique

By JOHN S. RUGGIERO and DONALD M. SKAUEN

Forty-seven ointment bases were evaluated for their ability to release radioactive sodium iodide when evaluated by a chick embryo technique. In general, ointments of the hydrophilic type indicated better release of the isotope than did bases of the absorption or oleaginous type.

A PREVIOUS REPORT (1) illustrates the development of a chick embryo technique which permits the investigator to evaluate the ability of various ointment bases to release radioactive sodium iodide. This chick embryo technique is employed here to determine the release of radioactive sodium iodide from different ointment bases. Limited studies were also conducted to evaluate the effect of surface-active agents and of varying quantities of water on the degree of absorption that might take place from ointment bases.

Received June 17, 1960, from the Research Laboratories, University of Connecticut, School of Pharmacy, Storrs.
Accepted for publication June 16, 1961.
Presented to the Scientific Section, A.PH.A., Los Angeles meeting, April 1958.

EXPERIMENTAL

Various ointment bases were selected as representatives of the three major categories of ointment bases as listed by Robinson (2), namely; those which are oleaginous or water repellent; those which absorb water, but are greasy and nonwashable; and those which are water miscible or water soluble.

Ointment Bases Selected.—*Oleaginous Bases.*—petrolatum U.S.P. XV; white petrolatum U.S.P. XV; yellow ointment U.S.P. XV; white ointment U.S.P. XV; lard N.F. X; Domolene, product of Dome Chemicals Inc.; Plastibase, product of E. R. Squibb and Sons; Spry, product of Lever Brothers, Inc.; Singiser base No. 200 (3); Singiser base No. 225 (3); Singiser base No. 425 (3); Singiser base No. 625 (3); 15% Epolene in liquid petrolatum, Epolene is a product of Eastman Chemical Products, Inc.

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Evaluation of the Absorption of Radioactive Sodium Iodide from Various Ointment Bases by Means of a Chick Embryo Technique

By JOHN S. RUGGIERO and DONALD M. SKAUEN

Forty-seven ointment bases were evaluated for their ability to release radioactive sodium iodide when evaluated by a chick embryo technique. In general, ointments of the hydrophilic type indicated better release of the isotope than did bases of the absorption or oleaginous type.

A PREVIOUS REPORT (1) illustrates the development of a chick embryo technique which permits the investigator to evaluate the ability of various ointment bases to release radioactive sodium iodide. This chick embryo technique is employed here to determine the release of radioactive sodium iodide from different ointment bases. Limited studies were also conducted to evaluate the effect of surface-active agents and of varying quantities of water on the degree of absorption that might take place from ointment bases.

Received June 17, 1960, from the Research Laboratories, University of Connecticut, School of Pharmacy, Storrs.
Accepted for publication June 16, 1961.
Presented to the Scientific Section, A.PH.A., Los Angeles meeting, April 1958.

EXPERIMENTAL

Various ointment bases were selected as representatives of the three major categories of ointment bases as listed by Robinson (2), namely; those which are oleaginous or water repellent; those which absorb water, but are greasy and nonwashable; and those which are water miscible or water soluble.

Ointment Bases Selected.—*Oleaginous Bases.*—petrolatum U.S.P. XV; white petrolatum U.S.P. XV; yellow ointment U.S.P. XV; white ointment U.S.P. XV; lard N.F. X; Domolene, product of Dome Chemicals Inc.; Plastibase, product of E. R. Squibb and Sons; Spry, product of Lever Brothers, Inc.; Singiser base No. 200 (3); Singiser base No. 225 (3); Singiser base No. 425 (3); Singiser base No. 625 (3); 15% Epolene in liquid petrolatum, Epolene is a product of Eastman Chemical Products, Inc.

TABLE I.—PER CENT ABSORPTION OF RADIOACTIVITY BY THYROID FOR EACH OINTMENT BASE

Ointment Base	Class	No. of Embryos Used	Average % Radioactivity in Thyroid	Standard Error or Mean
Hydrophilic ointment No. 1	H	45	2.837	0.477
Vanishing cream	H	19	1.610	0.400
Hydrophilic ointment No. 3	H	18	1.532	0.405
Multibase	H	16	1.246	0.475
Omnia cream	H	13	1.203	0.425
Hydrophilic ointment U.S.P. XIV	H	13	1.138	0.503
Polysorb	A	24	0.912	0.219
Unibase	H	12	0.877	0.326
Amerchol CAB	A	23	0.866	0.200
Hydrophilic ointment No. 5	H	22	0.736	0.219
o/w Emulsion base	H	11	0.728	0.261
Qualatum	A	13	0.668	0.233
Plastibase	O	57	0.638	0.138
Hydrosorb	A	15	0.508	0.162
Hydrophilic ointment U.S.P. XV	H	27	0.485	0.112
Beeler's base	H	19	0.472	0.124
Velvachol	H	10	0.394	0.145
Domolene	O	16	0.361	0.110
Polyethylene glycol ointment U.S.P. XV	H	18	0.309	0.099
Singiser base No. 250	A	11	0.304	0.143
Hydrous wool fat U.S.P. XV	A	29	0.262	0.033
Hydrophilic ointment No. 2	H	17	0.254	0.070
Hydrophilic ointment No. 4	H	20	0.229	0.065
Hydrophilic Plastibase	A	34	0.229	0.047
Rose water ointment U.S.P. XV	H	20	0.226	0.063
Singiser base No. 625	O	20	0.212	0.056
Hydrophilic petrolatum U.S.P. XV	A	19	0.193	0.060
15% Epolene in liquid petrolatum	O	12	0.193	0.075
Almay emulsion base	H	13	0.176	0.053
Singiser base No. 200	O	12	0.163	0.049
White petrolatum	O	17	0.139	0.036
Carbowax compound 1500	H	14	0.123	0.065
Lard N.F. X	O	15	0.122	0.026
Singiser base No. 225	O	20	0.109	0.029
Hydrosorb plus 40% water	A	17	0.109	0.032
Robinson's polyethylene ointment	H	11	0.091	0.030
Spry	O	18	0.089	0.026
Yellow ointment U.S.P. XV	O	16	0.087	0.030
Hydrosorb plus 20% water	A	20	0.075	0.020
Aquaphor	A	17	0.074	0.022
Falba	A	22	0.072	0.019
Singiser base No. 425	O	14	0.065	0.019
Petrolatum U.S.P. XV	O	14	0.061	0.030
White ointment U.S.P. XV	O	22	0.041	0.017
Hydrosorb plus 30% water	A	19	0.040	0.009
Wool fat U.S.P. XV	A	17	0.021	0.007
Hydrosorb plus 10% water	A	20	0.015	0.007

Absorption Bases.—Wool fat U.S.P. XV; hydrous wool fat U.S.P. XV; hydrophilic petrolatum U.S.P. XV; Aquaphor, product of Duke Laboratories; Hydrosorb, product of Abbott Laboratories; Hydrosorb plus 10% water; Hydrosorb plus 20% water; Hydrosorb plus 30% water; Hydrosorb plus 40% water; Polysorb, product of E. Fougera and Co., Inc.; hydrophilic Plastibase, product of E. R. Squibb and Sons; Qualatum, product of Almay, Inc.; Falba, product of Pfaltz and Bauer, Inc.; Amerchol CAB, product of American Cholesterol Products, Inc.; Singiser base No. 250 (3).

Hydrophilic Bases.—Rose water ointment U.S.P. XV; hydrophilic ointment U.S.P. XV; hydrophilic ointment U.S.P. XIV; polyethylene glycol ointment U.S.P. XV; hydrophilic ointment No. 1, contains all of the ingredients in hydrophilic ointment U.S.P. XV except that 0.5% Pluronic L64[1] was substituted for polyoxyl 40 stearate; hydrophilic ointment No. 2, as above with 1% Alrodyne 315;[2] hydrophilic ointment No. 3, as above with 1% Medialan LL33;[3] hydrophilic ointment No. 4, as above with 1% Deriphat 150A;[4] hydrophilic ointment No. 5, as above with 1% Deriphat XD160;[4] Velvachol, product of Texas Pharmacal Co.; Omnia cream, product of Dohme Chemicals, Inc.; Almay emulsion base, product of Almay, Inc.; Multibase, product of Ar-Ex Cosmetics, Inc.; Unibase, Product of Parke, Davis and Co.; Beeler's base (4); o/w emulsion base (5); vanishing cream (2); Carbowax compound 1500, product of Carbide and Carbon Chemicals Corp.; Robinson's polyethylene ointment (2).

For the most part, these bases were chosen because of the frequency with which they appear in dermatological preparations and also because of their availability. Some bases were chosen mainly on the basis of claims that have been made by various researchers (3–5).

Each ointment was prepared as described by Ruggiero and Skauen (1) and evaluated for its ability to promote penetration of radioactive sodium iodide through the shell membrane and chorioallantoic membrane to the thyroid glands of 11-day-old chick embryos. The percentage of

[1] Products of Wyandotte Chemicals Corp.
[2] Product of Alrose Chemical Co.
[3] Product of Antara Chemicals.
[4] Product of General Mills, Inc.

absorption that occurred in each case is reported in Table I.

Evaluation of the Effect of Water on Absorption.—The literature (6–8) reports some controversy with regard to the effect that water has in absorption from ointment bases. In order to check the efficiency of this new method in evaluating and distinguishing among bases according to their water content, a series of Hydrosorb bases was prepared, each of the bases containing different percentages of water by weight. The results of absorption from these bases is shown in Table II.

Evaluation of Surfactants in Hydrophilic Ointments.—Miller and Selle (9) report that wetting agents increase absorption by causing emulsification of sebum and making contact with the glandular and follicular cells of the skin. More recently, various researchers (10–12) have evaluated the release of medication from bases that contained various surface-active agents. In order to evaluate the efficiency of this new technique to distinguish among absorption from ointments that contain different surface-active agents, a series of hydrophilic ointments was prepared, each containing the basic formula for hydrophilic ointment U.S.P. XV except that the surface-active agent was changed in each case. The results of absorption from these bases is reported in Table III.

TABLE II.—PER CENT ABSORPTION OF RADIOACTIVITY FROM HYDROSORB CONTAINING VARIOUS AMOUNTS OF WATER

Base	No. of Embryos Used	Average % of Radioactivity in Thyroid
Hydrosorb	15	0.508
Hydrosorb plus 40% water	17	0.109
Hydrosorb plus 20% water	20	0.075
Hydrosorb plus 30% water	19	0.040
Hydrosorb plus 10% water	20	0.015

TABLE III.—EFFECT OF SURFACTANTS ON ABSORPTION FROM HYDROPHILIC OINTMENTS

Surf	No. of Embryos Used	Average % of Radioactivity in Thyroid
0.5% Pluronic L62 and 0.5% Pluronic L64	45	2.837
1% Medialan LL33	18	1.532
1% Sodium lauryl sulfate	13	1.138
1% Deriphat XD160	22	0.736
5% Myrj 52	27	0.485
1% Alrodyne 315	17	0.254
1% Deriphat 150A	20	0.229

SUMMARY AND CONCLUSIONS

Examination of Table I indicates that eight of the first 10 bases listed belong to the hydrophilic group, while no member of the hydrophilic group appears among the last ten bases. The mean percentage of absorption of radioactivity, calculated for all of the bases from each of the three ointment classes, indicates 0.969% absorption in the hydrophilic group and 0.294% and 0.248%, respectively, for the absorption and oleaginous groups. These figures indicate that absorption of sodium iodide was best from the hydrophilic group but that there was little difference between absorption from the oleaginous and absorption bases.

The ointment bases which contained polyethylene glycols showed low absorption percentages when compared to the other bases in the hydrophilic group, however, it should be noted that when these ointments were assayed, most of the chick embryos were dead before the thyroid glands were removed. Perhaps absorption had been so pronounced that death had come before the thyroids were able to concentrate more radioactive sodium iodide.

Singiser (3) has presented a series of bases that contain varying amounts of metallic soaps, liquid petrolatum, white petrolatum, and wool fat. Table I shows that of the Singiser bases which were evaluated, base No. 250, which contains 6% wool fat, is more capable of releasing radioactive sodium iodide than the other Singiser bases which were evaluated.

According to the results shown in Table II, the addition of water to Hydrosorb ointment base decreased the percentage of radioactive sodium iodide that was absorbed from the base itself. There does not seem to be any pattern established with regard to the degree of decrease and the amount of water added. This limited study indicates that the technique is capable of distinguishing among bases that contain varying percentages of water.

Results shown in Table III indicated that the chick embryo technique is capable of detecting differences in absorption of labeled sodium iodide from these ointments. The base which contains the 0.5% Pluronic L62 and 0.5% Pluronic L64 closely conforms to a formula prepared to contain the required hydrophilic-lipophilic balance for such a preparation.

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Study of the Boric Acid-Glycerin Complex II

Formation of the Complex at Elevated Temperatures

By JOHN J. SCIARRA and ANTHONY J. MONTE BOVI

A study of the boric acid-glycerin complex was conducted at temperatures of 30, 40, 50, and 60° and the effect of increasing the concentration of glycerin upon the solubility of boric acid was studied. This may then be indicative of the degree of complex formation taking place between these two substances. Concentrations of glycerin varying from 0 to 100% were utilized. The results of these experiments show the relationship between (*a*) solubility of boric acid and (*b*) temperature and concentration of glycerin. A phase diagram was constructed at these temperatures showing the quantitative relationship among boric acid, water, and glycerin. From a plot of the log solubility of boric acid *versus* the reciprocal of absolute temperature, the heat of solution at various concentrations of glycerin was calculated. It was found that the degree of complex formation between boric acid and glycerin increased with an increase in temperature but showed little, if any, increase with an increase in glycerin concentration from 0 to about 40%. However, above this concentration of glycerin, the degree of complex formation, as indicated by the solubility, increased with an increase in concentration of glycerin.

THE FORMATION of a complex compound between boric acid and glycerin has been known and investigated by many workers. This complex has been detected by many methods, among them being changes in pH (1, 2) and in solubility (3). In most cases the addition of glycerin to boric acid will allow the formation of a complex compound which is soluble to a greater extent than boric acid alone. However, during the course of previous investigations it has been found that boric acid behaves quite differently in glycerin than in the presence of other substances capable of forming a complex compound, such as sorbitol (4), mannitol, fructose, and other similar substances. Some of the differences in behavior between the boric acid-glycerin complex and the boric acid-sorbitol complex have been indicated in a previous publication (5) where the complex formation between these substances was studied at 25°. The results of this investigation prompted further work at elevated temperatures.

An increase in temperature generally will increase the solubility of substances in water and other solvents. The solubility of substances which are favorably affected by an increase in in temperature will show a negative heat of solution. The heat of solution of a substance will vary depending upon the concentration of solute in solution. The partial molal or differential heat of solution is defined as $d(\Delta H)/dn_2$, where ΔH is the heat of solution of a mole of solute in a quantity of solution so large that the addition of the one additional mole of solute does not appreciably change the concentration of the solution (6).

A solubility study can be used to determine the heat of solution of boric acid in varying concentrations of glycerin. By determining the solubility of boric acid in the various glycerin solvents at several temperatures and plotting these results, according to the van't Hoff relationship

$$\frac{d \ln S}{dt} = \frac{\Delta H}{2.303\ RT}$$

which when integrated becomes

$$\log S = \frac{-\Delta H}{2.303\ RT}$$

one can calculate the heat of solution. In the van't Hoff equation, S is the molal solubility of boric acid in the glycerin solvent, ΔH is the heat of solution, R is the gas constant, and T is the absolute temperature. An error is introduced since ΔH is treated as a constant, an assumption which is not entirely true (7).

Boroglycerin glycerite N.F. has been used for many years as an antibacterial substance. This consists chiefly of a 50% (by weight) solution of boroglycerin ($C_3H_5BO_3$) in glycerin. Official directions specify that this product be kept in a dry container (8). This glycerite is also described as a soluble preparation of boric acid (9), however, in certain cases, the addition of water is followed by the precipitation of boric acid. This is due to the hydrolysis of the boroglycerin complex to form boric acid which precipitates when water is not present in sufficient concentration.

Received April 28, 1961, from the Department of Pharmaceutical Chemistry, St. John's University, College of Pharmacy, Jamaica 32, N. Y.
Accepted for publication June 20, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

A comparison of this reaction with the reaction taking place between boric acid-sorbitol and water has previously been reported (5). However, to study this reaction more fully, the relationship between the amount of boric acid precipitated and the concentration of glycerin-water solutions was studied by combining various combinations of saturated solutions of boric acid in glycerin and in water at 25°.

EXPERIMENTAL

Saturated solutions of boric acid in solutions of glycerin and water were prepared in a manner previously reported (5). This method consisted of diluting glycerin U.S.P. containing 99%, by weight, of $C_3H_5(OH)_3$ with varying quantities of distilled water so that concentrations of glycerin were obtained from 0 to 100%, by weight, in increments of 10%. These solutions were then neutralized to phenolphthalein by the addition of a few drops of 1 *N* sodium hydroxide. An excess of finely divided, crystalline boric acid U.S.P. was added to about 100 ml. of glycerin solvent which had been placed into a 150-ml. glass solubility tube. The tube was placed into a constant temperature water bath at 30 ± 0.1° and stirred continuously for 24 hours (preliminary experiments showed that equilibrium had been attained during this period). After allowing the undissolved particles to settle to the bottom of the solubility tube, two 25-ml. portions of the clear, supernatant liquid were removed, placed into tared beakers, and quickly weighed. The amount of boric acid in each of these solutions was determined by assaying the solutions according to the U.S.P. assay for boric acid (10). The above procedure was repeated at temperatures of 40, 50, and 60°, all ±0.1°. Constant temperature was maintained through use of a Sargent full visibility, constant temperature water bath.

A different procedure was used to determine the amount of boric acid which would precipitate from saturated solutions of boric acid in both water and glycerin. A saturated solution of boric acid in water was prepared at 25° according to the official method (8), and assayed as above. A similar solution of boric acid was prepared in glycerin [over 99% by weight $C_3H_5(OH)_3$] and assayed. Various quantities of these solutions were added to a 150-ml. solubility tube in the quantities shown in Table I. Each tube contained a total weight of 100 Gm. These tubes were then tightly stoppered and placed into the constant temperature water bath at 25 ± 0.1° and allowed to stand without agitation. Maximum precipitation of boric acid was obtained at this time. Then two 25-ml. portions of the clear, supernatent liquid were removed and assayed as indicated above.

RESULTS

From the above results, the solubility of boric acid in the various glycerin-water solvents was calculated at elevated temperatures and expressed as weight per cent of boric acid as shown in Table II. In order to show the effect of temperature upon the solubility of boric acid Fig. 1 was constructed. Figure 2 relates the concentration of glycerin with the solubility of boric acid at the indicated temperatures.

The composition of each of the solutions was calculated on a weight per cent basis and the amount of each component present (boric acid-water-glycerin) is given in Table III. From this data, Fig. 3, which is a phase diagram of this multicomponent system, was constructed.

TABLE I.—PRECIPITATION OF BORIC ACID FROM SATURATED SOLUTIONS OF BORIC ACID IN GLYCERIN AND WATER

Saturated Solution H_3BO_3 in Glycerin, Gm.	Saturated Solution H_3BO_3 in Water, Gm.	H_3BO_3 in Glycerin Fraction, Gm.	H_3BO_3 in Water Fraction, Gm.	Total H_3BO_3, Gm.	Amount H_3BO_3 Found, Gm.	Amount H_3BO_3 Precipitated, Gm.	% H_3BO_3 Precipitated
100	0	16.68	0	16.68	16.68	0	0
97.5	2.5	16.26	0.12	16.38	16.40	0	0
95	5	15.85	0.24	16.09	16.06	0.03	0.19
90	10	15.01	0.48	15.49	15.17	0.32	2.07
85	15	14.18	0.72	14.90	12.13	2.77	2.28
80	20	13.34	0.96	14.30	9.72	4.58	32.03
75	25	12.51	1.20	13.71	8.12	5.59	40.77
72.5	27.5	12.09	1.32	13.41	7.73	5.68	42.36
70	30	11.68	1.44	13.12	7.20	5.92	45.12
65	35	10.84	1.68	12.52	6.44	6.08	48.56
60	40	10.01	1.92	11.93	6.05	5.88	49.29
55	45	9.17	2.16	11.33	5.68	5.65	49.87
50	50	8.34	2.41	10.75	5.37	5.38	50.05
45	55	7.51	2.65	10.16	5.16	5.00	49.21
40	60	6.67	2.89	9.56	5.12	4.44	46.44
35	65	5.84	3.13	8.97	4.90	4.07	45.37
30	70	5.00	3.37	8.37	4.63	3.74	44.68
25	75	4.17	3.61	7.78	4.66	3.12	40.10
20	80	3.34	3.85	7.19	4.72	2.47	34.35
15	85	2.50	4.09	6.59	4.72	1.87	28.38
10	90	1.67	4.33	6.00	4.73	1.27	21.17
5	95	0.83	4.57	5.40	4.74	0.66	12.22
2.5	97.5	0.42	4.69	5.11	4.78	0.33	6.46
0	100	0.00	4.81	4.81	4.81	0	0

TABLE II.—SOLUBILITY OF BORIC ACID IN GLYCERIN SOLUTIONS AT ELEVATED TEMPERATURES

Concentration of Glycerin, % w/w	Per Cent Weight Boric Acid 30°	40°	50°	60°
0	6.01	7.85	9.90	12.24
10	6.45	7.65	9.80	11.82
20	6.82	7.59	9.56	12.16
30	5.96	7.76	9.81	12.74
40	6.02	7.88	9.63	13.01
50	6.36	8.23	10.60	13.78
60	7.01	9.12	12.29	14.53
70	8.57	10.62	13.25	17.38
80	11.37	13.40	15.96	19.21
90	14.99	17.24	19.77	22.42
100	19.98	21.97	24.34	27.00

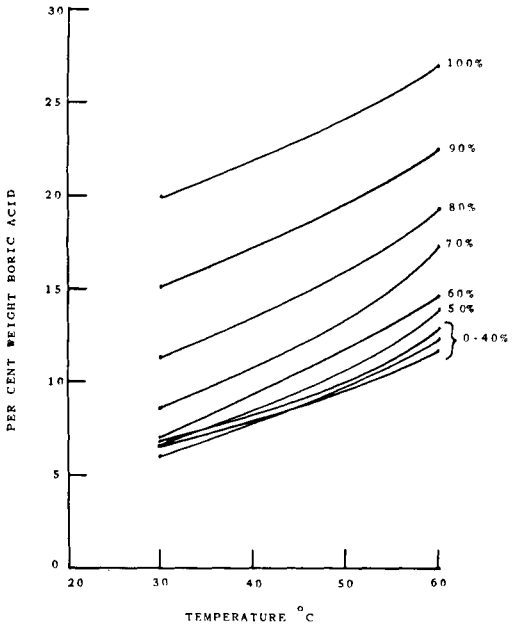


Fig. 1.—Effect of temperature upon the solubility of boric acid in glycerin.

Tables III and IV show the data necessary to plot Fig. 4 and calculate the heat of solution for each of the solvent systems studied. These also show the application of the van't Hoff equation to this system.

Table I shows the calculations involved in determining the amount of precipitation of boric acid taking place when saturated solutions of boric acid in both water and glycerin are combined.

These results are shown graphically in Fig. 5.

DISCUSSION

The degree of complex formation between boric acid and glycerin was found to increase with an increase in temperature as indicated by the corresponding increase in solubility of boric acid. The temperature effect also increased as the concentration of glycerin increased; that is, temperature had a greater effect at higher concentrations of glycerin. See Table V. This is shown in Fig. 1 where it can be noted that the curves representing from 0 to 40% are quite close to one another, in fact it is difficult to separate them. However, as one approaches

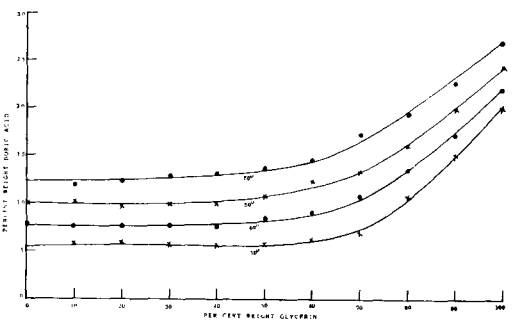


Fig. 2.—Isotherm curves showing solubility of boric acid in glycerin.

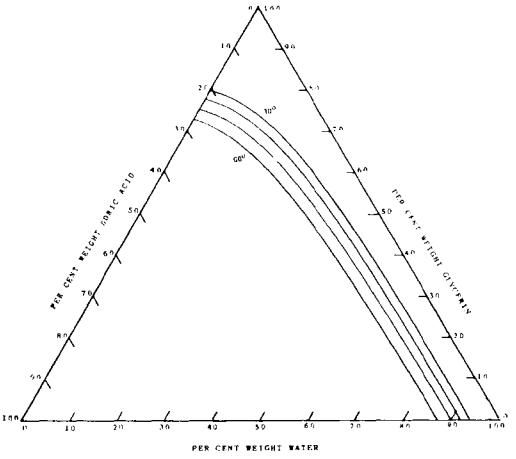


Fig. 3.—Phase diagram of boric acid-water-glycerin system.

50% and higher concentrations of glycerin, the curves are separated by a greater difference. The maximum temperature effect is obtained in going from a solvent containing 90% glycerin to one containing 100%. A similar relationship is found in Fig. 4 where the log solubility is plotted *vs.* reciprocal or absolute temperature. Since straight lines are produced in all cases, it may be assumed that the van't Hoff relationship is applicable to this system. Similar to Fig. 1, 0 to 40% glycerin concentrations show very little difference in solubility, as seen in Fig. 4. This difference becomes greater with increase in concentration of glycerin. In both Figs. 1 and 4 it can be seen that the temperature gradients going from 30 to 40°, 40 to 50°, and 50 to 60° seem to be about the same in all cases. This might be expected from results of prior reports. From Fig. 4, the slope can be determined from which the heat of solution can be calculated in each solvent. It is interesting to note that the slope of the lines for concentrations of glycerin from 30 to 60% are parallel, therefore they would have the same heat of solution. These results would indicate that very little, if any, complex formation takes place between boric acid and glycerin solutions in the range of 0 to 40%. This also seems to be independent of temperature.

A plot of the solubility of boric acid *vs.* concentration of glycerin, as shown in Fig. 2, also shows the

TABLE III.—COMPOSITION OF BORIC ACID-WATER-GLYCERIN SYSTEM AT ELEVATED TEMPERATURES

Composition of Solutions[a]											
30°			40°			50°			60°		
A	B	C	A	B	C	A	B	C	A	B	C
6.01	93.99	0	7.85	92.15	0	9.90	90.10	0	12.24	87.76	0
6.45	84.19	9.36	7.65	83.11	9.24	9.80	80.18	9.02	11.82	79.36	8 82
6.82	74.54	18.64	7.59	73.93	18.48	9.56	71.36	18.08	12.16	70.28	17.56
5.96	65.84	28.20	7.76	64.58	27.66	9.81	63.16	27.03	12.74	61.08	26.19
6.02	56.38	37.60	7.88	55.28	36.84	9.63	54.21	36.16	13.01	52.19	34.80
6.36	46.84	46.80	8.23	45.87	45.90	10.60	44.70	44.70	13.78	43.12	43.10
7.01	37.20	55.79	9.12	36.36	54.52	12.29	35.08	52.63	14.53	34.20	51.27
8.57	27.42	64.01	10.62	26.82	62.56	13.25	26.04	60.71	17.38	24.78	57.84
11.37	17.72	70.91	13.40	17.32	69.28	15.96	16.80	67.24	19.21	16.14	64.65
14.99	8.50	76.51	17.24	8.28	74.48	19.77	8.02	72.21	22.42	7.76	69.82
19.98	0	80.02	21.97	0	78.03	24.34	0	75.66	27.00	0	73.00

[a] A, Boric acid, % w/w; B, water, % w/w; C, glycerin, % w/w.

TABLE IV.—MOLAL SOLUBILITY OF BORIC ACID

Temperature[a]											
30°			40°			50°			60°		
A	B	C	A	B	C	A	B	C	A	B	C
63.94	1.0340	0.0145	85.19	1.378	0.1393	109.88	1.776	0.2495	139.47	2.255	0.3532
68.95	1.11	0.0453	82.84	1.340	0.1271	108.65	1.756	0.2435	134.04	2.167	0.3359
73.19	1.18	0.0719	82.13	1.328	0.1232	105.71	1.709	0.2326	138.43	2.238	0.3498
63.38	1.02	0.0086	84.13	1.360	0.1335	108.77	1.758	0.2450	146.00	2.360	0.3729
64.06	1.04	0.0170	85.48	1.382	0.1405	106.56	1.723	0.2362	149.55	2.418	0.3834
67.92	1.098	0.0407	88.71	1.435	0.1568	118.56	1.917	0.2826	159.82	2.584	0.4123
75.38	1.218	0.0856	99.26	1.605	0.2054	140.12	2.265	0.3551	170.00	2.749	0.4392
93.73	1.515	0.1804	118.81	1.921	0.2835	152.73	2.469	0.3925	210.36	3.401	0.5316
128.28	2.070	0.3160	154.73	2.502	0.3982	189.90	3.070	0.4871	237.77	3.844	0.5848
176.33	2.851	0.4550	208.31	3.368	0.5273	246.41	3.984	0.6003	288.99	4.673	0.6696
249.68	4.037	0.6061	281.55	4.552	0.6582	321.70	5.202	0.7162	369.86	5.980	0.7767

[a] A, Solubility of boric acid, Gm./1,000 Gm. solvent; B, solubility of boric acid, moles/1,000 Gm. solvent; C, log solubility.

TABLE V.—HEAT OF SOLUTION OF BORIC ACID-GLYCERIN SOLUTIONS

Concentration of Glycerin, % w/w	Heat of Solution Cal./Mole/° C.
0	−5,491
10	−4,576
20	−3,890
30	−5,720
40	−5,720
50	−5,720
60	−5,720
70	−5,034
80	−4,118
90	−3,203
100	−2,517

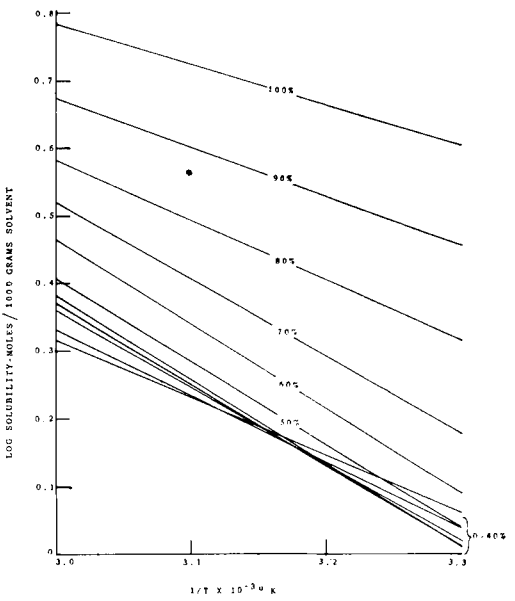


Fig. 4.—Application of data to van't Hoff equation.

lack of complex formation between these two substances until 45 or 50% concentrations of glycerin are attained. Then the solubility increases sharply as 100% glycerin is approached. A comparison of these results with the results obtained in studying the boric acid sorbitol complex are quite interesting (4). As previously indicated, the boric acid-sorbitol complex is formed in ranges from 0 to 70% sorbitol. This indicates increased stability of the boric acid-sorbitol complex as compared to the boric acid-glycerin complex, especially at low concentrations.

Temperature increases will give a greater area of miscibility to the boric acid-water-glycerin system as seen in Fig. 3. These curves are separated from one another by an equal distance, indicating the temperature effect to be constant.

In order to determine the amount of boric acid which would precipitate from solutions of the boric acid in glycerin (essentially boric acid-glycerin complex), varying quantities of a saturated solution of boric acid in water (to ensure that the amount of boric acid precipitated from the glycerin did not redissolve in water) was added to the glycerin solution of boric acid. These results are depicted in Fig. 5 where essentially a bell shaped curve is obtained.

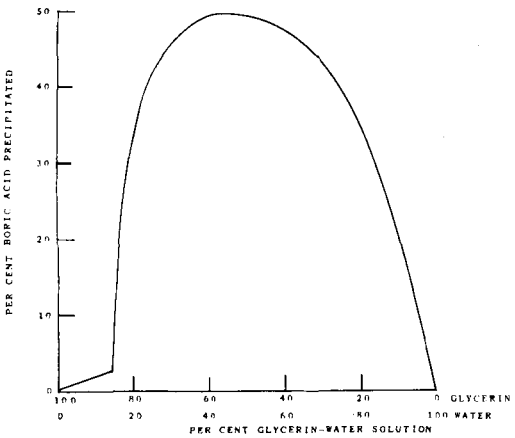


Fig. 5.—Precipitation of boric acid from glycerin-water solutions.

As expected, maximum precipitation of boric acid occurs about 45% water and 55% glycerin. About 15% water can be added without causing precipitation of boric acid. However, additional quantities of water will cause a large increase in the amount of precipitation. This observation would limit the use of boroglycerin glycerite when diluted with certain amounts of water.

SUMMARY AND CONCLUSIONS

1. A solubility study of the boric acid-water-glycerin system has been made at temperatures of 30, 40, 50, and 60°.

2. The degree of complex formation between boric acid and glycerin was found to increase with an increase in temperature.

3. Concentrations of glycerin from 0 to 40% had little effect, if any, upon the degree of complex formation as indicated by a solubility determination. This complex was found to increase as the concentration of glycerin was increased from 40 to 100%.

4. The heat of solution for each of the solvent systems was calculated through use of the van't Hoff relationship.

5. Maximum precipitation of boric acid from solutions of boric acid in glycerin was found to take place at a concentration of 45% water and 55% glycerin.

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Infrared Method for the Determination of Ethanol and Acetone in Thimerosal Tincture N.F.

By FREDERIC A. GRONAU, DONALD E. BROADLICK, and JAMES E. HAMILTON

The distillation method for determining ethanol in thimerosal tincture N.F. is time consuming and involves special handling to prevent interference of the acetone in the preparation. A shorter method is described based on the extraction of the ethanol into carbon tetrachloride and the measurement of the infrared absorption at 11.37 μ. The relative freedom from the interference by other alcohols and acetone assures greater specificity than the distillation method. The acetone present in tincture thimerosal N.F. can also be determined using the same extracted solution by reading the absorption of the carbonyl band at 5.86 μ.

THE DETERMINATION of ethanol in thimerosal tincture N.F. as described in the National Formulary (1) is that of Thorpe and Holmes (2). It has the disadvantage of being time consuming. It is also possible that it may not give a true estimate of the amount of ethanol in the preparation due to incomplete recovery of the ethanol in the distillate. The method formerly used in this laboratory was a modification of that of Hoff and Macoun (3) using paraformaldehyde and sodium hydroxide to form a nonvolatile reaction product with the acetone before distilla-

Received April 28, 1961, from the Analytical Department, Eli Lilly and Co., Indianapolis, Ind.
Accepted for publication June 23, 1961.
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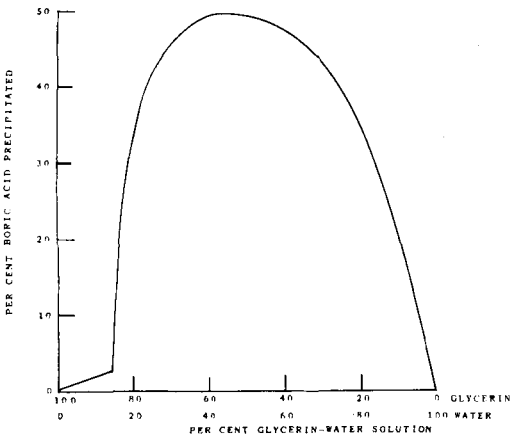


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tion. The ethanol content is then determined by measuring the specific gravity of the distillate. In an attempt to overcome these disadvantages, an infrared spectrophotometric method has been developed. It has proved to be faster, more accurate, and is specific for ethanol.

Souder and Deluca (4) reported the determination of chloroform in pharmaceuticals by infrared absorption measurement. This procedure has been adapted to the determination of ethanol in tincture thimerosal. The method involves extraction of the ethanol from the sample into carbon tetrachloride. The carbon tetrachloride is dried and the absorption measured at an ethanol band at 11.37 μ. Although not of primary concern, the acetone content of the sample can be determined on the same extracted solution by measuring the absorption of the carbonyl band at 5.86 μ.

EXPERIMENTAL

Band Selection.—The infrared spectra of the common pharmaceutical solvents used in external preparations—ethanol, acetone, methanol, and isopropanol—were obtained on a Beckman IR-5 recording spectrophotometer. The ethanol band at 11.37 μ was chosen for quantitative estimation for several reasons. This unique band does not seem to be associated with the hydroxyl portion of the molecule (5) which eliminates the interference of many compounds such as other alcohols. The band appears in a generally nonabsorptive region of the spectrum where the common solvents listed show little absorption (Fig. 1). The band also follows Beer's law in the concentration range expected to be found in the tincture thimerosal N.F., that is, $\pm 10\%$ of the theoretical amount. In this region of the spectrum, carbon tetrachloride shows very little absorption.

General Technique.—To a suitable glass-stoppered flask containing exactly 50 ml. of carbon tetrachloride, 1 ml. of the tincture is added. About 30 Gm. of anhydrous sodium sulfate is added to form a hydrate with the water in the sample, allowing more of the ethanol to be dissolved in the carbon tetrachloride. The mixture is shaken for 1 minute. Several anhydrous salts capable of binding the water were tried and sodium sulfate was found to be the most suitable. By removing water in this manner one may transfer 98%+ of the ethanol into the carbon tetrachloride. About 5 ml. of the extracted solution is removed and dried in a suitable flask with about 2 Gm. of anhydrous sodium sulfate. This is a precautionary measure to protect the sodium chloride cells. The absorption of this solution is then determined at 11.37 μ in 1.0-mm. sodium chloride cells against a blank of carbon tetrachloride. After correcting for any cell blank, the absorption is compared to that of a standard run in the same manner. A Beckman model IR-2 spectrophotometer with sodium chloride optics was used for all quantitative results reported here.

Standard Preparation.—Standards were prepared in two ways. The alcohol and acetone were removed from a sample of tincture thimerosal by evaporating under vacuum until the absorption maximum of ethanol at 11.37 μ and of acetone at 5.86 μ disappeared. This blank material was then reconstituted by adding it to exactly 50 ml. of absolute ethanol and 10 ml. of acetone in sufficient volume to make 100 ml. A mixture of water, ethanol, and acetone was prepared by diluting 50 ml. of absolute ethanol and 10 ml. of acetone to 100 ml. with water. The absorption of carbon tetrachloride extracts of these solutions was determined at both the ethanol band at 11.37 μ and at the acetone band at 5.86 μ and found to be identical. All results reported in this work are determined against the water-ethanol-acetone standard. It became apparent from the work done on the standards that acetone could be determined on the same solution by reading the carbonyl band at 5.86 μ. Although the tincture contains only one-fifth as much acetone as ethanol, the unusually strong absorption of the carbonyl band resulted in an absorption almost as great as that of ethanol.

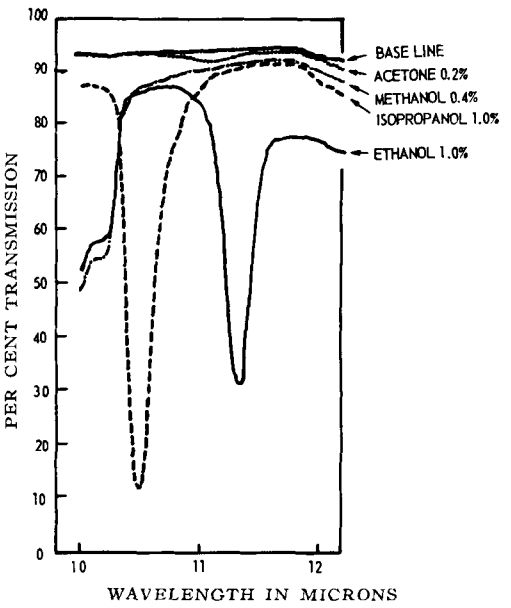


Fig. 1.—Infrared spectra of ethanol, methanol, acetone, and isopropanol in carbon tetrachloride.

Comparison of Methods.—Ten different production lots of the tincture were assayed using the general technique. These results are compared to those obtained by distillation (Table I). In the distillation procedure, 50 ml. of the tincture is diluted with 20 ml. of saturated sodium chloride solution in a liter boiling flask. A 7-Gm. quantity of paraformaldehyde and 5 ml. of a 50% solution of sodium hydroxide are added. This mixture is then distilled in a compact distilling apparatus using Friedichs condensers. About 50 ml. of distillate is collected and diluted to 100 ml. The specific gravity is measured on a Westphal balance and the per cent ethanol obtained from Standard Alcoholimetric Tables found in the U.S.P.

The per cent acetone found in the samples is included in Table I but the comparison to other methods is not made. It is noted that the infrared

TABLE I.—COMPARISON OF ETHANOL DETERMINATION BY INFRARED AND DISTILLATION

Sample	Distillation Ethanol, %	Infrared Ethanol, %	Infrared Acetone, %
1	48.30	49.17	9.31
2	47.16	49.69	9.50
3	47.50	50.22	9.60
4	47.66	50.22	9.75
5	48.92	49.28	9.73
6	48.30	49.48	9.55
7	48.62	50.43	9.58
8	48.46	49.48	9.63
9	48.30	50.63	9.70
10	48.62	49.70	9.47
Average	48.18	49.83	9.58
Theoretical	50.00	50.00	10.00

TABLE II.—REPRODUCIBILITY OF INFRARED ASSAY

	Ethanol, %	Acetone, %
Run 1		
	50.32	9.87
	50.22	10.13
	50.32	10.00
	50.53	10.08
Run 2		
	50.85	9.98
	50.22	9.98
	50.53	9.87
	50.53	9.98
Average	50.44	9.99
Standard deviation (S.D.)	0.208	0.090
3 × S.D., %	1.25	2.70

method gives results which are closer to the theoretical amounts of ethanol in the product. An attempt was made to explain the higher results found by the infrared method. It had been felt that the distillation apparatus in use did not give complete recovery of the ethanol in the product. Using a system of added internal standard, the recovery efficiency of the distillation method for ethanol and the efficiency of the infrared method for both ethanol and acetone were measured.

Absolute ethanol was added to a previously assayed sample and the ethanol content was redetermined. By subtracting the original ethanol result from the total and comparing this figure to the amount of ethanol added, the efficiency of recovery was found. This study shows the distillation procedure used recovers only 96.7% of the ethanol while the infrared technique recovers 99.8%. This difference of 3.1% is consistent with that of 3.29% experienced in assaying the 10 samples by each method. The same technique was applied to the acetone which also shows a 99.8% efficiency.

Reproducibility of Method.—One sample was assayed in two groups of four aliquots each. These results are shown in Table II. The estimated standard deviation on these data was found to be 0.208 or about 0.4%. The reproducibility for these eight samples (3 × S.D.) was ±1.25%. The acetone standard deviation is 0.09 or about 0.9%. The reproducibility is ±2.7%.

SUMMARY

An infrared method is presented for the determination of ethanol in thimerosal tincture N.F. The method is less time consuming than distillation procedures and gives results which are closer to the theoretical amounts of ethanol in the product. The acetone in the product may also be determined in the same sample. Additional data are being collected on other hydroalcohol preparations and will be included in subsequent manuscripts.

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Structural Studies on Terebinthone from *Schinus terebinthefolius*

By KRISHAN K. KAISTHA and LEMONT B. KIER

A new triterpene, terebinthone, has been isolated from the berries of *Schinus terebinthefolius* (Radd.). Analyses indicate the formula $C_{30}H_{46}O_3$. Spectral evidence indicates that the compound is a diketone with one group in the C-3 position and the second in a highly hindered position. Reduction and hydrogenation studies supported these findings. Ozonolysis produced acetaldehyde showing that the single double bond is in the side chain as an ethylidene group. The third oxygen of terebinthone is believed to be in a cyclic ether ring.

THE TREE *Schinus terebinthefolius* (Radd.), also known as the Brazilian pepper tree, belongs to the family *Anacardiaceae*. It is a small evergreen tree found in south and central Florida. During the winter months numerous clusters of small, bright red berries appear (1). These berries are said to exert a paralyzing effect on birds upon ingestion.

This study was initiated in an effort to evaluate the principle producing this action, to identify it as well as other major isolable products chemically, and to determine if there may be anything of therapeutic value. In the course of the study, the berries were initially defatted with hexane. From this solvent there was obtained a crystalline substance, which, though not possessing the aforementioned pharmacology, still was of considerable interest chemically. This compound is the subject of this first communication.

Analyses of this compound, provisionally named terebinthone, and several derivatives indicated that the molecular formula $C_{30}H_{46}O_3$ best fit the data. Further confirmation of this formula was found in the closeness of the Rast molecular weight to the theoretical value.

It was concluded that terebinthone was a triterpene from the finding of four C-methyl groups, gem dimethyl and terminal methyl group absorption in the infrared (1385 and 1365 cm.$^{-1}$) (2, 3), the close similarity in the infrared spectra of terebinthone and its derivatives with other triterpenes, color tests, and the molecular formula.

Perbenzoic acid titration (4) indicated the presence of one double bond. Infrared analysis showed two ketonic carbonyl group absorptions at 1708 and 1690 cm.$^{-1}$. The remaining oxygen function did not appear as a carbonyl or hydroxyl group in the infrared spectrum, but an absorption at 1095 cm.$^{-1}$ was observed in terebinthone and several other derivatives which has been assigned to the C—O—C group of a cyclic ether (5, 6).

The presence of two carbonyl groups and one double bond indicated that terebinthone is basically tetracyclic, and with the cyclic ether would be pentacyclic. This conclusion is in agreement with the molecular formula.

A closer examination of the carbonyl groups indicated that the peak at 1708 cm.$^{-1}$ could be ascribed to an isolated aliphatic or isolated six-membered ring ketone (3, 7). Terebinthone showed no absorption in the conjugated carbonyl region of the ultraviolet, confirming the fact that both keto groups are isolated. It was concluded that the 1708 cm.$^{-1}$ absorption peak was due to a 3-keto group from the fact that terebinthone gave a positive Zimmermann reaction (8) and an absorption maximum at 367 mμ for the mono-2,4-dinitrophenylhydrazone derivative. Reich (9) has shown that absorption in the 367–369 mμ region is due to the presence of this derivative of a 3-keto group.

The 1703–1690 cm.$^{-1}$ region is reported (2, 10) to be due to a saturated keto group in a hindered position. This is in agreement with the absorption of the second carbonyl group of terebinthone (1690 cm.$^{-1}$). The fact that this group was hindered was shown by the fact that only a mono-2,4-dinitrophenylhydrazone could be formed even under forcing conditions. This derivative still showed the 1690 cm.$^{-1}$ absorption.

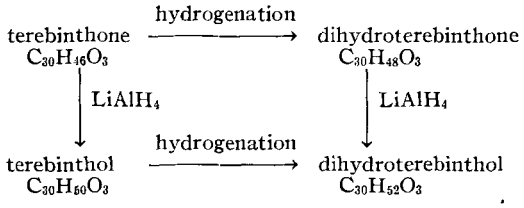
Reduction of terebinthone with lithium aluminum hydride (11) gave a diol, terebinthol, $C_{30}H_{50}O_3$. This compound had only one acylable function, forming a mono-3,5-dinitrobenzoate, confirming the presence of a highly hindered keto group in terebinthone. The 1095 cm.$^{-1}$ absorption (cyclic ether) indicated that no change had occurred in the ether ring. Terebinthol gave a positive Liebermann-Burchard reaction (12) indicative of a 3-hydroxyl group.

Hydrogenation of terebinthone to dihydro-

Received April 28, 1961, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Florida, Gainesville.
Accepted for publication June 6, 1961.
Taken from a thesis by Krishan K. Kaistha submitted in partial fulfillment of the requirements for the degree of Ph.D.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

terebinthone, $C_{30}H_{48}O_3$, with the measured consumption of hydrogen equivalent to one double bond, confirmed the presence of one reactive double bond in the molecule. Dihydroterebinthone gave a positive Zimmermann reaction and formed only a mono-oxime even under vigorous conditions (13). The 1095 cm.$^{-1}$ absorption was present indicating no cleavage of this ring.

Hydrogenation of terebinthol and lithium aluminum hydride reduction of dihydroterebinthone gave the same compound, dihydroterebinthol, $C_{30}H_{52}O_3$.



The compound retained the 1095 cm.$^{-1}$ absorption peak (cylic ether) in the infrared. A mono-tosylate was prepared which confirmed the fact that all four of these compounds contain a highly hindered oxygen function.

Zaffaroni (14) described a technique useful for partial characterization of steroids which consists of measurement of absorptions of concentrated sulfuric acid solutions from 200 to 500 mμ. The principal maxima for the four compounds above was at 304 mμ. Absorption between 280 and 296 mμ is associated with an α,β-unsaturated ketone (15). The fact that all four compounds exhibited the same maxima clearly indicates that no change has occurred in the positions of the oxygen functions and that the double bond, when present, does not exhibit any effect on any of these.

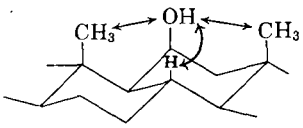
The Wolff-Kishner reduction (16) of dihydroterebinthone gave a major product with a molecular formula of $C_{30}H_{50}O_2$. The infrared analysis showed absorption at 1095 cm.$^{-1}$ (cyclic ether) and 1703 cm.$^{-1}$ which must be due to the same hindered carbonyl group previously discussed, since a successful Wolff-Kishner reduction depends upon the formation of a phenylhydrazone intermediate and no derivatives could be made of one carbonyl group. Furthermore, this compound, 3-desoxy-dihydroterebinthone gave no Zimmermann reaction, indicating that the 3-keto group had been reduced.

There can be no question that the hindrance of one oxygen function in these compounds is of the magnitude of the C-11 position in steroids or the C-7 and C-11 positions of tetracyclic triterpenes. In the latter case, the C-7 carbonyl group of lanostane-7,11-dione-3-yl acetate (I) has been reduced by the Wolff-Kishner method (17) to the 11-monoketone (II). Though this does not



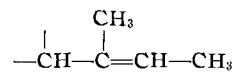
(I) (II)

exclude the possibility of a C-7 keto group in terebinthone, or other possibilities as well, the evidence strongly suggests that the hindered keto group might well be in the C-11 position. If reduction of this keto group occurs to give an 11β-hydroxyl group, then this would account for the failure to form a derivative of this function. The 11β-hydroxyl group is practically unacylable (18) due to steric strain from two angular methyl groups and a hydrogen (III).



(III)

Ozonolysis (19) of terebinthone gave a volatile compound identified through its 2,4-dinitrophenylhydrazone as acetaldehyde. It appeared likely from this evidence that the double bond was located in the side chain as an ethylidene group. In an attempt to learn more of the environment of this unsaturation, terebinthone was treated with dry hydrogen chloride (20) to induce rearrangement if possible. The product, which could not be purified, was treated with ozone as before. The volatile product, identified through a derivative, was methyl ethyl ketone. From these reactions it is possible to conclude that the side chain must possess the arrangement (IV).



(IV)

In regard to the location of the cyclic ether function, no evidence is yet available though studies are continuing in this laboratory.

EXPERIMENTAL

All melting points were taken on the Kofler apparatus unless otherwise stated. Analyses were performed by the Weiler and Strauss Microanalytical Laboratory, Oxford, England, and Galbraith Laboratories, Knoxville, Tenn. Spectral work was done by the Department of Chemistry, University of Florida.

Plant Material.—The ripe berries of *Schinus terebinthefolius* (Radd.) were gathered during the winter months of 1959–1960 and 1960–1961 near St. Petersburg, Fla.[1] A voucher specimen of the plant is deposited in the herbarium of the University of Florida. The berries were air-dried for 3 days then minced in a meat grinder.

Isolation of Terebinthone.—Nine hundred grams of the minced berries was extracted with hexane in a Soxhlet extractor for 24 hours. The hexane was gently expelled under an air stream leaving a brown, viscous oil weighing 117 Gm. A crystalline deposit gradually formed in the oil and several crops were filtered from it. An average of 4 Gm. of crystalline material was obtained from several extractions or about 0.45% of the crude berries. The crude material was recrystallized several times from acetone and finally from alcohol to give fine needles, m.p. 178–179°, $[\alpha]_D^{22}$ −72.6° (chloroform). Chromatography of the material on a Celite column did not improve these values. The name terebinthone was provisionally assigned to this compound.

Anal.—Calcd. for $C_{30}H_{46}O_3$: C, 79.24; H, 10.20; mol. wt., 454; C-methyl, 13.3 (four). Found: C, 79.22; H, 10.22; mol. wt. (Rast), 445, 456; C-methyl, 12.7.

Infrared analysis indicated absorption at 1708 and 1690 cm.$^{-1}$ (two carbonyls), 1640 cm.$^{-1}$ (double bond), 1385 and 1365 cm.$^{-1}$ (gem dimethyl and angular methyl) and 1095 cm.$^{-1}$ (cyclic ether). Ultraviolet analysis indicated absorption at 213 mμ (E = 7272). The compound gave a positive Zimmermann test and a negative test with tetranitromethane. The material was insoluble in hot sodium hydroxide solution.

Terebinthone Mono-2,4-dinitrophenylhydrazone.—A solution of 60 mg. of terebinthone in 10 ml. of aldehyde-free alcohol was treated with 25 ml. of U. S. P. 2,4-dinitrophenylhydrazine solution and refluxed for 4 hours. The product was isolated in the usual way and recrystallized from ethanol-ethyl acetate to give orange needles, m.p. 221–223°.

Anal.—Calcd. for $C_{36}H_{50}N_4O_6$: C, 68.11; H, 7.94; N, 8.83. Found: C, 68.75; H, 7.57; N, 9.19.

Ultraviolet analysis indicated maximum absorption at 367 mμ (E = 1409). Infrared analysis showed absorption at 1690 cm.$^{-1}$ (carbonyl).

Perbenzoic Acid Titration of Terebinthone.—A 40-mg. sample of terebinthone was dissolved in purified chloroform at 5° and made up to exactly 10 ml. with the same solvent. Exactly 9 ml. of this solution was withdrawn and added to exactly 5 ml. of a solution of perbenzoic acid in benzene at 5°. The mixture was swirled and kept at 5° for 24 hours. A blank was prepared using 9 ml. of purified chloroform. At the end of 24 hours, a 2-ml. aliquot was added to a solution of 1 Gm. potassium iodide, 1 ml. acetic acid, and 20 ml. of water. The liberated iodine was titrated with 0.01 *N* sodium thiosulfate. The perbenzoic acid consumed was equivalent to 0.98 double bond. At the end of 48 hours, the acid consumption was equivalent to 1.1 double bonds.

Ozonolysis of Terebinthone.—A solution of 200 mg. of terebinthone in 25 ml. of carbon tetrachloride at 0° was treated with a stream of 3% ozonized oxygen for 30 minutes. Water was then added to the reaction mixture and the volatile constituents were then steam distilled into a solution containing 200 mg. of 2,4-dinitrophenylhydrazine, 14 ml. of 40% sulfuric acid, and 6 ml. of water. About half of the carbon tetrachloride was steam distilled to insure completeness of the distillation. The distillate mixture was shaken and the organic layer separated. The aqueous phase was then extracted with ether which was added to the carbon tetrachloride solution. The residue from this solution was recrystallized from ethanol to give needles, m.p. 143–145°. A mixed melting point with the corresponding acetaldehyde derivative gave no depression and the infrared spectra were identical.

Acid Treatment of Terebinthone.—A solution of 400 mg. of terebinthone in 30 ml. of distilled chloroform was treated with a slow stream of dry hydrogen chloride for 7 hours. The hydrogen chloride and the chloroform were then removed under vacuum leaving an oily residue which could not be crystallized. This residue was then treated with ozone as previously described. The 2,4-dinitrophenylhydrazone obtained, m.p. 104–106°, gave no depression in melting point when mixed with the corresponding derivative of methyl ethyl ketone.

Dihydroterebinthone.—A solution of 1 Gm. of terebinthone in 30 ml. of ethyl acetate was hydrogenated at 1 atmosphere using prereduced platinum oxide as a catalyst. The consumption of hydrogen was equivalent to 92% of the theoretical amount for one double bond. The product from evaporation of the ethyl acetate was recrystallized from methanol to give 940 mg. of needles, m.p. 152–153.5°, $[\alpha]_D^{22}$ −80.8° (chloroform).

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.89; H, 10.59. Found: C, 78.83; H, 10.78.

Infrared analysis indicated strong absorption at 1700 cm.$^{-1}$ (carbonyl). The compound gave a positive Zimmermann test.

Dihydroterebinthone Mono-oxime.—A solution of 225 mg. of dihydroterebinthone and 650 mg. of hydroxylamine hydrochloride in 25 ml. of dry pyridine was refluxed for 20 hours. The pyridine was removed under vacuum and the residue recrystallized from methanol-water to give microcrystals, m.p. 164–166°.

Anal.—Calcd. for $C_{30}H_{49}NO_3$: C, 76.38; H, 10.47; N, 2.97. Found: C, 76.20; H, 10.62; N, 3.26.

Infrared analysis indicated absorption at 1700 cm.$^{-1}$ (carbonyl).

Terebinthol.—A suspension of 400 mg. of lithium aluminum hydride in 30 ml. of dry, peroxide-free ether was stirred for 1 hour. To this was added dropwise a solution of 500 mg. of terebinthone in dry, peroxide-free ether. Stirring was continued for 24 hours. At the end of this time, 1 ml. of 10% sodium hydroxide solution was added dropwise, followed by 1.2 ml. of water, then stirring continued for another 18 hours. The reaction mixture was then treated with water and the ether phase separated. The residue from the ether was recrystallized from acetone-hexane to give prisms, m.p. 189.5–191.0°, $[\alpha]_D^{22}$ −49.1° (chloroform).

Anal.—Calcd. for $C_{30}H_{50}O_3$: C, 78.55; H, 10.99;

[1] The authors wish to thank Mr. Lloyd Remington and his students at Boca Ciega High School for gathering the material. We are also indebted to them for introducing to us the local folklore concerning this plant's action.

active hydrogen (two), 0.41. Found: C, 79.08; H, 10.59; active hydrogen, 0.39.

Infrared analysis indicated a strong absorption at 3400 cm.$^{-1}$ (hydroxyl) and 1035 cm.$^{-1}$ (C—O). There was no absorption in the carbonyl region. The compound gave positive Liebermann-Burchard and Salkowski tests but negative Tortelli-Jaffe and tetranitromethane tests.

Terebinthol Mono-3,5-dinitrobenzoate.—A solution of 100 mg. of terebinthol and 500 mg. of 3,5-dinitrobenzoyl chloride in 3 ml. of dry pyridine was heated for 1 hour on a steam bath then allowed to stand at room temperature for 5 hours. This solution was then added to 10 ml. of water and the precipitate filtered off and washed with 2% sodium carbonate and water. The crude material was chromatographed on an alumina-Celite (1:2) column. The lower of two bands, developed with benzene, was cut from the column and extracted with chloroform. The residue from the chloroform was recrystallized from methanol, m.p. 119–121°.

Anal.—Calcd. for $C_{37}H_{52}N_2O_8$: C, 68.07; H, 8.03; N, 4.29. Found: C, 68.59; H, 7.74; N, 4.58.

Infrared analysis indicated absorption at 3400 cm.$^{-1}$ (hydroxyl).

Dihydroterebinthol.—(*a*) *Hydrogenation of Terebinthol.*—A solution of 1.7 Gm. of terebinthol in ethyl acetate was hydrogenated as previously described. The residue from the ethyl acetate was chromatographed on alumina. One gram of product was eluted with ether and recrystallized from methanol to give rods, m.p. 159–161°, $[\alpha]_D^{22}$ −38.7° (chloroform).

Anal.—Calcd. for $C_{30}H_{52}O_3$: C, 78.20; H, 11.38; Found: C, 78.49; H, 11.27.

(*b*) *Reduction of Dihydroterebinthone.*—An ethereal solution of 350 mg. of dihydroterebinthone was reduced with lithium aluminum hydride as previously described. This product, m.p. 159–161°, gave no depression in melting point with the compound obtained by the hydrogenation of terebinthol. The infrared spectra were also identical.

Dihydroterebinthol Mono-tosylate.—A solution of 100 mg. of dihydroterebinthol and 600 mg. of *p*-toluenesulfonyl chloride in 5 ml. of pyridine was heated on a water bath for 4 hours. The solution was then cooled, diluted with aqueous acid, and extracted with ether. The ether solution was washed with 5% sodium carbonate, dried, and the residue recrystallized from methanol to give needles, m.p. 144–146°.

Anal.—Calcd. for $C_{37}H_{56}O_5S$: C, 72.54; H, 9.15; S, 5.23. Found: C, 72.24; H, 9.40; S, 4.99.

Infrared analysis indicated absorption at 3400 cm.$^{-1}$ (hydroxyl).

Wolff-Kishner Reduction of Dihydroterebinthone (3-Desoxy-dihydroterebinthone).—Two hundred milligrams of sodium in 50 ml. of distilled, dry diethylene glycol was heated to 180°, 2.4 ml. of anhydrous hydrazine was added, and refluxing continued for 5 minutes. The solution was then cooled to 140° and 1 Gm. of dihydroterebinthone added, followed by 5 ml. of diethylene glycol. Refluxing was resumed with intermittent shaking to dissolve the compound completely. Refluxing was continued for 22 hours at 180°, then some hydrazine was allowed to escape until the refluxing temperature rose to 210°. This temperature was maintained for another 22 hours. The reaction mixture was cooled, diluted with water, and extracted with benzene. The residue from the benzene weighed 50 mg. and could not be further purified.

The aqueous phase was acidified with 10% acetic acid and extracted with benzene. The residue from the benzene, weighing 200 mg., was recrystallized from ethanol-water to give crystals, m.p. 137–139°, $[\alpha]_D^{22}$ −37.8° (chloroform).

Anal.—Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38; mol. wt., 442. Found: C, 81.10; H, 11.33; mol. wt. (Rast), 444.

Infrared analysis showed a single carbonyl absorption (1703 cm.$^{-1}$) and cyclic ether (1095 cm.$^{-1}$). The compound gave no Zimmermann reaction.

Absorption Spectra in Sulfuric Acid.—The absorption spectra between 220 and 700 mμ of concentrated sulfuric acid solutions of terebinthone, terebinthol, dihydroterebinthone, and dihydroterebinthol were obtained after time intervals of 5, and 15 minutes, 2 and 24 hours. The principal maxima in each case was observed at 304 mμ. A secondary peak in the terebinthone solution at 258 mμ was observed to disappear with the passage of time.

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Production of Cardiac Glycosides by Plant Tissue Cultures I

Nutritional Requirements in Tissue Cultures of *Digitalis lanata* and *Digitalis purpurea*

By E. JOHN STABA

The techniques employed and the agar medium suggested for the establishment of *Digitalis lanata* Ehrh. and *Digitalis purpurea* L. seed and root callus tissue are reported. Particular attention is devoted to the concentration effects of the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), 2-benzothiazoleoxyacetic acid (BTOA), and 1-naphthaleneacetic acid (NAA) contained in a growth medium suggested for the two *Digitalis* species.

THE GENERAL procedures employed to establish *in vitro* plant tissue cultures have been well documented and reported (1–3). However, the establishment of a defined medium for a specific plant to be grown as either normal or callus tissue is still determined empirically.

Nickell (4), Steward (5), and Bergmann (6) have shown that callus plant tissue cultures could be transferred to liquid submerged cultures and grown as single plant cells or small aggregates of plant cells. Many investigators have since speculated on the possible application of plant tissue cultures for producing economically useful plant constituents (4, 7).

The experimental evidence pertaining to the capability of an *in vitro* plant tissue culture to biosynthesize a desired plant constituent is inconclusive. *Datura* crown-gall tumors are known to contain high amounts of hyoscine (7); *Datura tatula* L. root tissue cultures are known to biosynthesize the alkaloids hyoscyamine and hyoscine (8); and, *Atropa belladonna* L. root and callus tissue cultures are known to biosynthesize the alkaloid atropine (9). However, callus tissue cultures of *Agave toumeyana* Trel. leaves do not contain the sapogenin hecogenin (10).

This publication represents the first one of a series that will attempt to determine whether or not tissue cultures of cardiac glycoside-containing plants can biosynthesize cardiac glycosides.

EXPERIMENTAL

The digitalis seeds used on medium A (see Table I) were obtained from the Saier Seed Co., Dimondale, Mich., and those seeds used on medium B were obtained from the medicinal plant laboratory and garden, University of Minnesota.[1] Roots were obtained from digitalis plants approximately 6 months old and growing in the drug plant greenhouse, University of Nebraska.

Sterilization of Seeds and Roots.—Digitalis seeds were sterilized in a 5.25% sodium hypochlorite solution (Purex Corp., Ltd., South Gate, Calif.) diluted 1:4 with sterile distilled water for approximately 10 minutes in vacuum. The seeds were then washed with sterile distilled water and transferred to sterile Petri dishes containing approximately 10 ml. of sterile distilled water. Two seeds were transferred aseptically to a nutrient medium after approximately 80% of the seeds had begun to germinate. This occurred on the fifth day for *D. lanata* Ehrh. seeds and on the third day for *D. purpurea* L. seeds.

Root sections were cut from digitalis plants grown in soil, washed in tap water, and sterilized in the above solution in vacuum for approximately 15 minutes. By aseptic procedures, the sterilized root sections were washed with distilled water, placed in Petri dishes containing a small amount of distilled water, and chopped with a scalpel into small root sections of about 2 cm. The root sections were then transferred aseptically to a nutrient medium.

Auxin Stock Solutions and Coconut Milk.—All auxins were prepared as 30% ethanol stock solutions. The auxins and coconut milk were autoclaved with the constituents of medium A or medium B. With the exception of 2-benzothiazoleoxyacetic Acid (BTOA),[2] all auxin stock solutions were used within 48 hours of their preparation.

Experiment One.—Medium A, with 0.7% agar, was prepared in the following four variations: (*a*) medium A; (*b*) medium A with 2,4-dichlorophenoxyacetic acid (2,4-D), 2 parts per million (p.p.m.); (*c*) medium A without enzyme-hydrolyzed casein hydrolysate (EHCH) but with 2,4-D, 2 p.p.m.; and (*d*) medium A without EHCH, coconut milk, or 2,4-D. The pH after autoclaving of media (*a*) and (*b*) was 4.1, and of media (*c*) and (*d*), 5.4. The low pH of media (*a*) and (*b*) was due to the presence of EHCH and caused the media to liquefy.

Received April 28, 1961, from the College of Pharmacy, University of Nebraska, Lincoln.
Accepted for publication July 18, 1961.
Supported in part by grant from the National Heart Institute (H-5308) and the University of Nebraska Research Council, Grants-in-Aid (Johnson Fund—1960).
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
The author is indebted to Mr. Sidney J. Stohs for technical assistance.

[1] Courtesy of the late Dr. Earl B. Fischer, Professor of Pharmacognosy, University of Minnesota.
[2] Supplied through the courtesy of the Organic Chemical Research Section, Lederle Laboratories, Pearl River, N. Y.

TABLE I.—DIGITALIS TISSUE CULTURE MEDIA

Medium A[a]		Medium B[a]	
Coconut milk, %	15.0	Coconut milk, %	15.0
Sucrose,[b] %	2.0	Sucrose,[b] %	2.0
meso-Inositol, %	0.5	Modified White's inorganic salts, mg./L.	
Vitamin-free, enzyme-hydrolyzed casein, %	0.2	$MgSO_4$	360.0
Murashige's inorganic salts,[c] mg./L.		$Ca(NO_3)_2$	200.0
KNO_3	1,900.0	Na_2SO_4	200.0
NH_4NO_3	1,440.0	KNO_3	80.0
$MgSO_4 \cdot 7\ H_2O$	370.0	KCl	65.0
$CaCl_2$	332.0	NaH_2PO_4	16.5
KH_2PO_4	136.0	$MnSO_4$	4.5
$MnSO_4 \cdot 1\ H_2O$	14.0	$ZnSO_4$	1.5
$ZnSO_4 \cdot 7\ H_2O$	8.1	H_3BO_3	1.5
H_3BO_3	4.8	KI	0.75
KI	0.8	Na_2MoO_4	0.021
$(NH_4)_6MoO_2 \cdot 4\ H_2O$	0.18	$CuSO_4$	0.002
$CuSO_4 \cdot 5\ H_2O$	0.02	Iron-ethylenediamine tetraacetic acid[d]	50.0
Iron-ethylenediamine tetraacetic acid[d]	79.5	Vitamins solution[e] (same as medium A)	
Vitamins solution[e]		Simple supplement	
Biotin	1.0	*meso*-Inositol	200.0
Choline	1.0	Glycine, anhydrous	2.0
Folic acid	1.0	Niacin	0.4
Nicotinamide	1.0		
Pantothenic acid	1.0		
Pyridoxal	1.0	pH Adjustments made with ammonium hydroxide solution	
Thiamine	1.0		
Riboflavin	0.1		

Seitz-Filtered Complex Supplement for Medium B

	mg./L.		mg./L.
Glucose-1-phosphate	100.0	Chlorogenic acid	10.0
L-Glutamine	100.0	Methyl-β-L-arabinopyranoside	5.0
L-Glutamic acid	50.0	Adenosine triphosphate disodium salt	5.0
L-Aspartic acid	50.0	Adenine (hemi) sulfate	5.0
Urea	50.0	Desoxyribonucleic acid	3.0
L-Arabinose	50.0		
L-Asparagine	25.0		

[a] *Media* were prepared with inorganic chemicals of reagent grade, organic chemicals from either Nutritional Biochemical Corp., Cleveland, Ohio, or Calbiochem, Los Angeles, Calif., and distilled water, redistilled from glass. [b] Pfanstiehl Laboratories, Waukegan, Ill. [c] Dr. Toshio Murashige, University of Hawaii, formerly in the laboratory of Dr. Folke Skoog, University of Wisconsin. [d] Perma Green Iron 135, Refined Products Corp., Lyndhurst, N. J. [e] Eagle's vitamins solution, Hyland Laboratories, Los Angeles, Calif.

Murashige's mineral solution; Murashige's mineral solution, half-strength; and modified White's mineral solution were prepared with each of the above four variations.

Two sterile, germinated digitalis seeds were placed aseptically on 5 ml. of each medium contained in 17 mm. × 100 mm. sterile plastic test tubes.[3]

Experiment Two.—Medium B, with 0.7% agar, was prepared in the following three variations: (*a*) medium B without coconut milk; (*b*) medium B with coconut milk and EHCH, 0.2%; and (*c*) medium B with coconut milk and complex supplement.

To each of the above three media, auxins were added to yield the following p.p.m. concentrations: indoleacetic acid (IAA) 0.1 and 1; BTOA 2 and 6; 2,4-D 4 and 8; naphthaleneacetic acid (NAA) 6; BTOA 2 + IAA 0.1; BTOA 2 + 2,4-D 4; and IAA 0.1 + 2,4-D 4 + NAA 2. The following auxin concentrations also were used in medium (*a*): BTOA 2 + kinetin 0.5; and IAA 0.1 + kinetin 0.5.

The complex supplement was Seitz-filtered and, at all times, added aseptically to autoclaved and cooled medium B.

The above media were adjusted to pH 5.7 with ammonium hydroxide solution before Seitz-filtration or autoclaving.

Experiment Three.—Medium B was prepared in the following two variations: (*a*) medium B, and (*b*) medium B with complex supplement.

In each of the above two media, auxins were used in the p.p.m. concentrations shown in Table II and in the following auxin combinations: BTOA 1 + 2,4-D 5; BTOA 5 + 2,4-D 1; BTOA 5 + 2,4-D 5; BTOA 1 + NAA 1 + 2,4-D 5.

Approximately 15 ml. of each medium in experiment 2 and experiment 3 was added to autoclaved, 1-oz., plastic-capped, wide-mouth prescription bottles. Two sterile, germinated digitalis seeds were placed aseptically in each bottle. This procedure was repeated four times for each medium.

RESULTS AND DISCUSSION

Experiment One.—Germinated digitalis seeds were completely inhibited in growth if placed on medium A, or any of its variations containing

[3] Falcon Plastics, Los Angeles 45, Calif.

TABLE II.—RELATIVE GROWTH OF DIGITALIS SEED CALLUS ON MEDIUM B CONTAINING COMPLEX SUPPLEMENT[a]

Auxin Concn., p.p.m.	*Digitalis purpurea*, Days of Growth			*Digitalis lanata*, Days of Growth		
	25	65	130	35	80	115
BTOA						
0.5	0	0	0	0	0	+
1.0	0	0	+	0	+	+
5.0	0	+	++	0	+	+++
10.0	0	+	+++	0	+	+++
2,4-D						
1.0	+	++	+++	+	++	+++
2.0	+	++	++++	+	+	+++
5.0	+	++	++	+	++	+++
10.0	+	+	++	+	+	++
NAA						
1.0	0	0	0	0	0	+[b]
2.0	0	0	+	+	+	+
5.0	0	+	+	+	++	++
10.0	+	+	++	+	++	++

[a]Of 16 control Digitalis plants, one developed a small callus after 95 days of growth. [b]Of eight plants, one developed a callus on the 115th day.

EHCH. Although this substance is normally a good nutrient supplement for plant tissue cultures (11), it has affected tissue growth of Jerusalem artichoke tubers adversely in the absence of nitrate ions (12). Casein hydrolysate, in the absence of IAA and kinetin, also caused adverse growth effects in tobacco callus cells (13).

Germinated digitalis seeds grew normal-appearing roots and stems on medium A without EHCH, coconut milk, or 2,4-D. Root development was generally poor unless the medium contained IAA or NAA. One small callus appeared after approximately 45 days of growth on medium A without EHCH, but with 2,4-D, 2 p.p.m.

No significant influence on the growth of digitalis seeds by any of the three mineral solutions used was observed.

Experiment Two.—Medium B was prepared at a pH of 5.7 with EHCH in order to confirm that this substance was responsible for the inhibition of germinated digitalis seeds and not the high acidity of the medium. As previously observed in experiment 1, all germinated digitalis seeds in experiment 2 were also completely inhibited in growth on media containing EHCH.

Due to the adverse growth effects of EHCH, a chemically defined complex supplement (see Table I) was formulated to serve as the nitrogen and carbohydrate source. This supplement appeared to increase the vigor of digitalis seed growth, with the one exception of media containing IAA where it appeared to retard root development.

Small callus tissues were observed to form occasionally from sterile, germinated digitalis seeds or roots on media containing the auxins, 2,4-D, BTOA, or their combinations. Media containing NAA stimulated root production. Previous investigators have obtained questionable growth effects when digitalis was grown on a medium containing 2,4-D, 6 p.p.m., with NAA, 0.1 p.p.m. (1).

Experiment Three.—As the digitalis seeds consistently grew better on a medium with the complex supplement, the descriptions of growth given below and in Table II pertain only to media containing the complex supplement.

Digitalis seeds developed normal-appearing stems and leaves on media without auxins for about 65 days (Fig. 1, frame 1), with eventual drying and browning of the stem and leaves after about 115 days (Fig. 2, frame 2). Root systems in the control plants were poorly developed.

Effects of the Auxin, BTOA.—The stimulatory effects of BTOA on tissue growth of both carrot and Jerusalem artichoke tuber have been reported (11).

D. purpurea appeared slightly less sensitive (Fig. 1, frames 4, 5, 6) and more varied (Fig. 1, frame 2) in response to BTOA than *D. lanata* (Fig. 2, frames 4, 5, 6). The *D. purpurea* callus shown in Fig. 1, frame 2 is growing on medium B with BTOA, 5 p.p.m., and increased more than 6,000% when calculated as the wet weight over the original weight of the seed (0.1 mg.) in 95 days.

In both digitalis species callus appeared to form from the stem-root node, stem, or cotyledon tissue in contact with the medium.

Effects of the Auxin, 2,4-D.—All digitalis seeds on media containing 2,4-D formed very poor stems and roots (Fig. 2, frame 1) and eventually formed callus tissues.

Both digitalis species at the lower 2,4-D concentrations, developed excellent callus tissue within 25 to 35 days (Fig. 1, frames 7, 8, 9). The callus would generally develop from small nodules on either the roots, stems, or cotyledons (Fig. 2, frames 7, 8, 9). Root-like nodules were observed to form on some callus tissues of *D. lanata* (Fig. 2, center of frames 8, 9).

Effects of the Auxin, NAA.—*D. purpurea* seeds at the lower NAA concentrations generally produced good stem growth and a short, thick root system (Fig. 1, frame 3). At the higher NAA concentrations, the seeds developed practically no stems and were transformed into small callus tissues containing small rootlets (Fig. 1, frames 10, 11, 12).

D. lanata seeds at the lower NAA concentrations generally produced good stem growth, a short, thick root system, and occasionally callus tissue. At the higher NAA concentrations all the *D. lanata* seeds developed a rapidly growing callus containing an abundance of root nodules and root hairs (Fig. 2, frames 10, 11, 12). In contrast to the growth rate of *D. purpurea* tissue, the growth of *D. lanata* tissue on higher NAA concentrations was rapid.

Effects of Combinations of the Auxins, BTOA, 2,4-D, and NAA.—Digitalis seeds formed callus tissue on all auxin combinations containing 2,4-D. However, if the 2,4-D concentration was 5 p.p.m. or greater, the callus growth was generally less vigorous. Combinations of BTOA, 1 or 5 p.p.m., with 2,4-D, 1 p.p.m., resulted in very good callus growth formation for both digitalis species (Figs. 1 and 2, frames 13, 14, 15).

Digitalis Root Callus Formation.—The incidence of contamination in bottles containing digitalis roots of plant origin was about 50%. Callus tissue did occasionally form on medium B containing either BTOA, 2,4-D, NAA, or their combinations. *D. lanata* root callus formation is shown on medium B containing NAA, 10 p.p.m. (Fig. 2, frame 3).

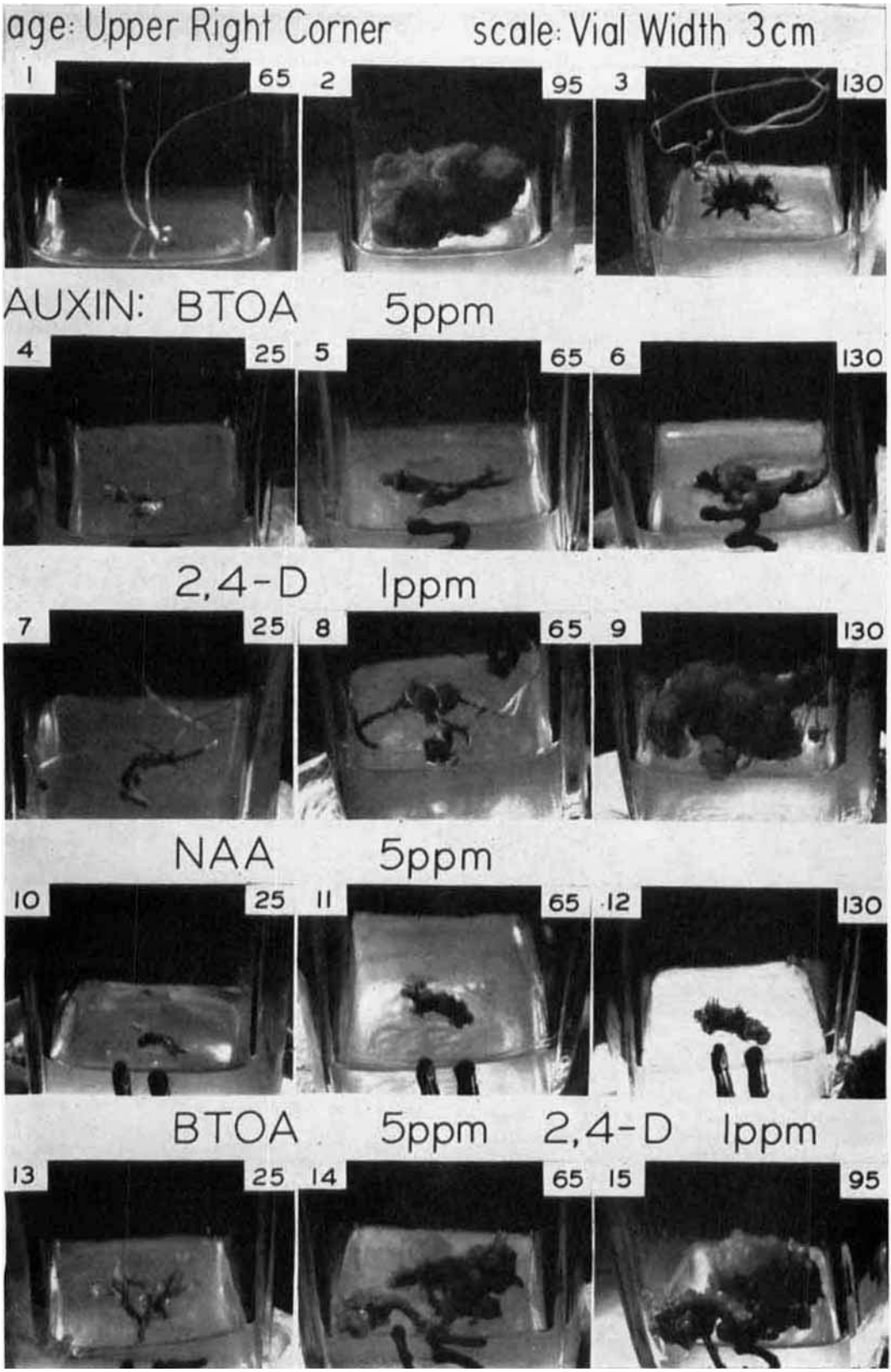


Fig. 1.—*Digitalis purpurea* L.

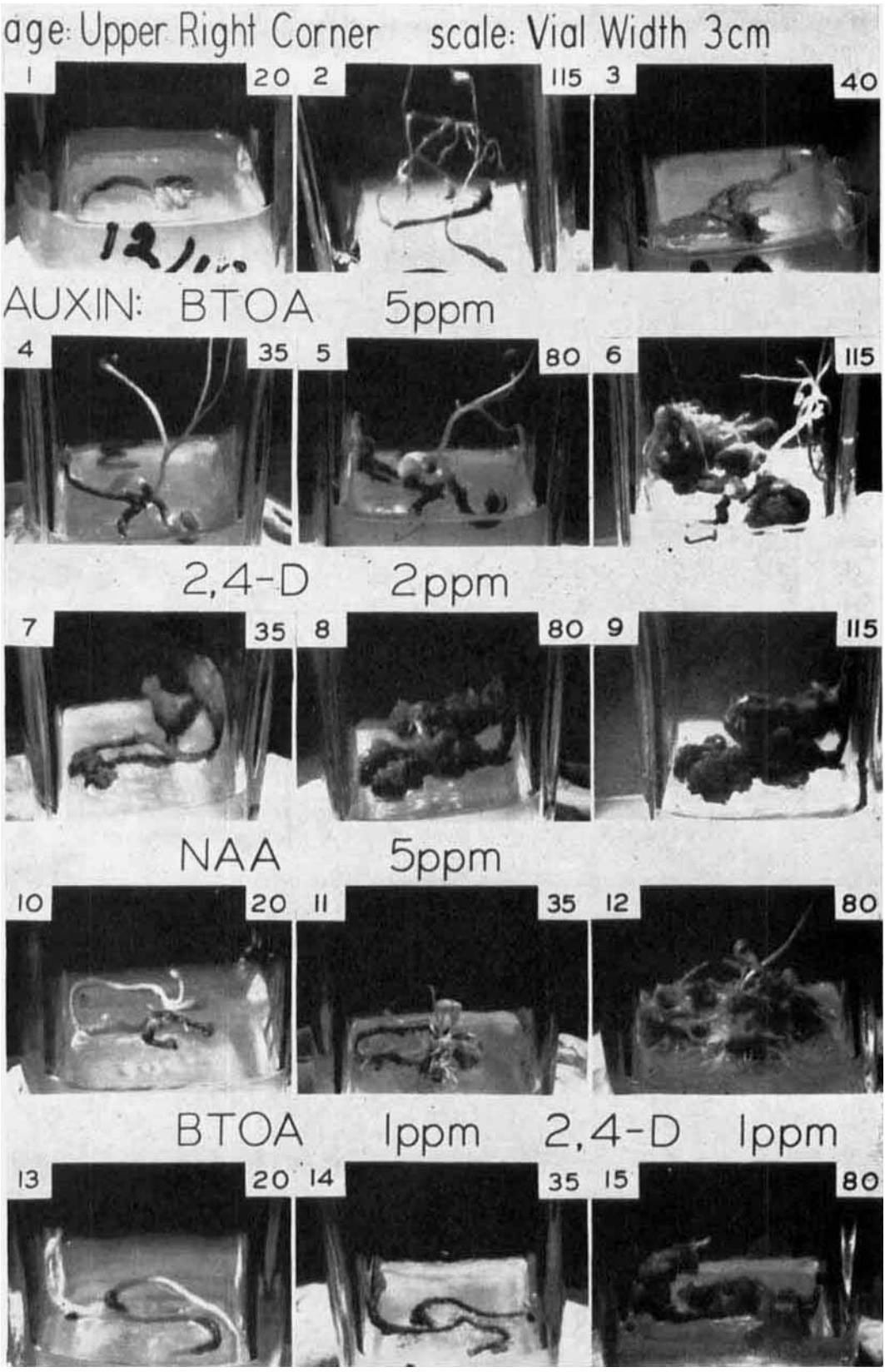


Fig. 2.—*Digitalis lanata* Ehrh.

SUMMARY AND CONCLUSIONS

1. On the media defined, enzyme-hydrolyzed casein will inhibit the growth of germinated digitalis seeds.

2. On medium B with complex supplement, the auxins BTOA, 5 p.p.m.; 2,4-D, 2 p.p.m.; NAA, 5 p.p.m.; or appropriate auxin combinations, BTOA, 1 to 5 p.p.m., with 2,4-D, 1 to 2 p.p.m., will cause digitalis seeds or roots to form callus tissues.

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Radioisotopic Method of Evaluating Dispersed Systems I

Emulsions

By JAMES B. APPINO†, JOHN E. CHRISTIAN, and GILBERT S. BANKER

Liquid petrolatum emulsions were prepared by a standard method and evaluated by a new radioisotopic method. The radioisotopic method required less time and was more sensitive than previously used methods. A radioisotopic creaming rate was determined for each emulsion and can be used to compare microscopically identical emulsions and/or production samples of an emulsion to a previously determined standard. The method is applicable to w/o or o/w emulsions.

THE PRECISE evaluation of emulsions has presented problems to pharmaceutical and other scientists for many years. In an attempt to appraise and control emulsion appearance, uniformity of dosage, and stability, the following emulsion properties have been evaluated: particle size, particle size distribution, viscosity, and creaming rate (1–3). The evaluation of these factors gives some insight into the question, "How long can the product remain pharmaceutically elegant on the pharmacist's shelf?" but may leave the question of uniformity either wholly or partially unanswered.

This study is concerned primarily with an evaluation of the creaming rate and/or phase uniformity of emulsions in less time than is now required by the conventional visual (4) or microscopic (5) methods, or the more tedious though perhaps more accurate chemical or analytical methods (6). Radioisotopes, because of their sensitivity of detection (7), were utilized as tools in evaluating these properties.

Received April 28, 1961, from Purdue University, School of Pharmacy, Lafayette, Ind.

Accepted for publication June 12, 1961.

Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.

† Present address: Armour Pharmaceutical Co., Kankakee, Ill.

EXPERIMENTAL

Preparation of Emulsion Samples.—The compositions of the emulsions evaluated in this study are given in Table I. The standard method of manufacture of all emulsion samples involved heating the liquid petrolatum and emulsifiers to 55° and the water and preservatives to 60°. The water phase was then added to the oils with constant agitation and the emulsion mixed until the temperature dropped to 38°. All emulsions were prepared in 1-L. quantities and allowed to deaerate for 24 hours before use.

Approximately 1 mc.[1] of sodium iodide (I^{131})[2] or iodobenzene (I^{131}) (8) per 100 ml. of emulsion was

[1] Gave 8,000–12,000 c.p.m. in end counting and 5,000–8,000 c.p.m. in side counting apparatus. Apparatus described later in text.

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TABLE I.—EMULSION FORMULAS AND IDENTITY KEY

Ingredients, %	Emulsion Numbers[a] 1	2	3
Liquid petrolatum (heavy)	25.0	25.0	25.0
Span 20	1.2	4.2	1.8
Tween 20	0.8	2.8	1.2
Sodium iodide	..	0.5	..
Paracepts concentrate	1.0	1.0	1.0
Distilled water	72.0	66.5	71.0

[a] Emulsion 1: 1A, NaI^{131} added to finished emulsion; 1A', replication of 1A. Emulsion 2: 2A, NaI^{131} added to finished emulsion; 2B, $C_6H_5I^{131}$ added to finished emulsion. Emulsion 3: 3A, NaI^{131} added to finished emulsion; 3B, $C_6H_5I^{131}$ added to finished emulsion; 3C, $C_6H_5I^{131}$ added before emulsification.

used to label the emulsions studied. These labels were chosen on the basis of their selective solubilities in the water and oil phases, respectively, and insolubility in the other components of the emulsions. One of the following methods was used to prepare any single emulsion sample: (*a*) NaI^{131} was added to the water phase of the finished emulsion, (*b*) iodobenzene (I^{131}) was either added to the oil before emulsification, or (*c*) incorporated in 2 ml. of liquid petrolatum, 0.5 ml. of Span 20,[3] and 0.5 ml. of Tween 20[3] and added to the finished emulsion.

To facilitate detection of any visual changes in phase uniformity of the emulsions, 5–10 drops of a 1% amaranth solution was added to 120 ml. of an unlabeled control sample and to 120 ml. of a labeled control sample of each emulsion.

Evaluation of Emulsion Samples.—Approximately 120 ml. of each emulsion was placed in a separate 18 mm. i.d., constant bore Pyrex cell, 44 cm. in length. Gentle suction was then applied with a water aspirator for approximately 3 minutes to remove any air remaining in the emulsion. The cells were filled to within 3 mm. of the top and one cell was then placed in the counting apparatus and counted. Three other cells containing unlabeled, uncolored; unlabeled, colored; and labeled, colored emulsions, respectively, were stored as controls.

Two counting apparatuses were constructed to determine the ratioactivity in the labeled emulsions (Fig. 1). The first apparatus, number 1, was designed to detect activity through the side of the cell and the second apparatus, number 2, through the ends of the cell. Initially, the side counting method appeared to be the logical method to use in evaluating the emulsions; but due to its insensitivity when the water phase was labeled, the end counting method proved to be superior.

In the end counting apparatus the cells were sealed with Saran[4] wrap and paraffin. The Saran wrap was placed over the end of the cell and paraffin poured around the outer edge to seal it (no paraffin was used at the upper end of the cell). Two Geiger-Müller tubes, type D-33,[5] were used to detect the activity in the cell. The upper tube was placed against a 1/8 inch thick lead plate that had a 1/4 inch diameter hole drilled through it. The lower tube was placed through a plywood platform drilled just to accommodate the tube with the end window against another lead plate which had a 1/4 inch diameter hole drilled through it for collimation.

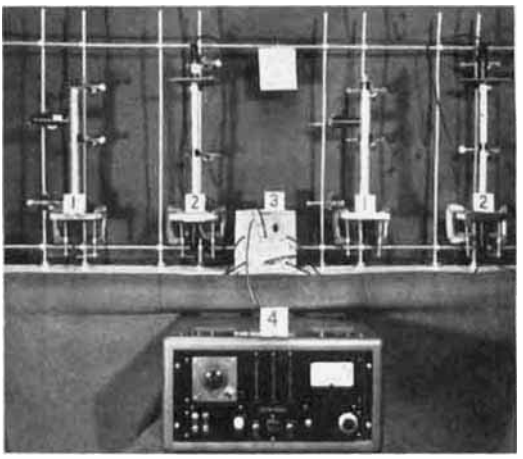


Fig. 1.—Multiple counting apparatus. 1, Side counting apparatus; 2, end counting apparatus; 3, control panel; 4, scaler.

The cell was placed on a 1.5 × 2.5 inch piece of hard rubber to aid in sealing the bottom. The rubber had a 15 mm. diameter hole cut in it and the cell was positioned above this hole. The plywood platform was supported by a support ring attached to a permanent table mounted ring stand. Each Geiger-Müller tube was held in a fixed position by a ring stand clamp.

The cables from six Geiger-Müller tubes were connected to a central control panel (number 3, Fig. 1). The scaler, model 200-S[6] (number 4, Fig. 1), was also connected to this panel and by turning the panel switch from 1 through 6, each of the Geiger-Müller tubes could be put into operation without manually changing any connections.

Viscosity and Particle Size Determination.—A direct microscopic evaluation (9) of the globule diameters was made using a standard microscope equipped with a 10× eyepiece and 43× objective. The eyepiece contained a micrometer with a 5 mm. horizontal scale divided into 100 divisions. A 50% aqueous solution of propylene gylcol was used to dilute one drop of the emulsion three to fivefold to facilitate microscopic examination (10). One drop of the diluted emulsion was placed on a microscope slide, a cover slip placed over it, and then allowed to stand 15 minutes before examination. A sample size of 100 particles was found to be more than statistically adequate using the method of Harris, Horvitz, and Moon (11), with a maximum confidence interval of one unit, a 99% confidence coefficient, and a 95% probability.

The emulsions used in this study were thought to exhibit Newtonian properties (12). This supposition was verified using a Ferranti-Shirley cone-plate viscometer.[7] A Brookfield-Synchro-lectric, model RVF viscometer[8] was thus used in subsequent viscosity determinations using spindle No. 1 at 20 r.p.m. The viscometer was allowed to operate 10 minutes in the emulsion before a reading was taken.

[3] Atlas Powder Co., Wilmington 99, Del.
[4] Dow Chemical Co., Midland, Mich.
[5] Nuclear-Chicago Corp., Des Plaines, Ill.
[6] Radiation-Instrument Development Laboratory, Chicago 21, Ill.
[7] Ferranti Ltd., Manchester 10, England.
[8] Brookfield Engineering Laboratories, Stoughton, Mass.

TABLE II.—STATISTICAL DATA AND RADIOISOTOPIC CREAMING RATES

Emulsion	Correlation Coefficients[a] r_1	r_2	r_3	Radioisotopic Creaming Rate[b]	Last Significant Day[c]	Visual Creaming[d]
1A	0.8772	0.9213	0.9912	0.2047	1	10[e]
1A′	0.9080	0.9550	0.9912	0.2074	1	10[e]
2A	0.8881	0.9358	0.9970	0.3596	4	4[e]
2B	0.9898	0.9750	0.9948	0.8238	7	6
3A	0.9588	0.9513	0.9648	0.0947	1	None
3A Hourly	0.9203	0.9751	0.9992	0.1441	..	None
3B	0.9052	0.9740	0.9920	0.0860	1	None
3B Hourly	0.9626	0.9546	0.9961	0.1382	..	None
3C	0.9542	0.9536	0.9974	0.1506	1	1
3C Hourly	0.9441	0.9023	0.9910	0.2957	..	None

[a] r_1 = Zero order, r_2 = first order, r_3 = log-log. [b] Rate of change of ratio with respect to time, based on log-log plot. [c] Last day significantly different from all other days (Newman-Keuls test). [d] Day phase separation first observed in control emulsions. [e] No distinct phase separation, only a change in color intensity of the controls.

Statistics.—An analysis of variance (13) was used to identify significant differences in phase uniformity of the emulsion samples with time and a Newman-Keuls test (14) was employed to determine the days over which significant changes occurred. The method of least squares (13) was used in the graphical presentations of the data and correlation coefficients (13) were calculated for zero-order, first-order, and log-log expressions to determine the best form of expression of the data.

RESULTS

Radiation Effects.—When the particle size and viscosity data of the various emulsions were statistically analyzed, it was found that the addition of NaI^{131} or $C_6H_5I^{131}$ to the finished emulsion, in the manner described and in the amounts used, had no effect on the emulsion system.

Radioisotopic Evaluation.—The radioisotopic data were recorded as a ratio of counts per minute (c.p.m.) at the top of the cell to c.p.m. at the bottom of the cell. By using this ratio, a form of comparison counting was achieved and corrections for background, efficiency, absorption, and decay were unnecessary. This left only coincidence corrections to be applied to the data. The length of each count was the time calculated to give 1% error or less in counting, usually 5 minutes. The emulsions were counted three times a day at 6-hour intervals until the ratio no longer decreased. The data were then statistically analyzed and plotted. The plots of emulsions 1A, 2A, and 3A are given in Fig. 2. These are typical graphs for emulsions with the water phase labeled. The graphs for the emulsions with the oil phase labeled were of the same type except the slopes were positive.

Two cells of an identical emulsion (1A and 1A′) were used to determine if reproducible results could be achieved on different apparatus. After the counting was completed, an analysis of variance was conducted to determine if day to day differences did exist and a Newman-Keuls test (14) was run to determine which days differed significantly. In both emulsions there were significant day to day differences, but the Newman-Keuls test indicated no significant differences after the first day.

To determine the rate of change of the ratio with respect to time (radioisotopic creaming rate)[9] the

[9] The radioisotopic creaming rate is the rate of phase separation or creaming of labeled emulsions (ratio *vs.* time).

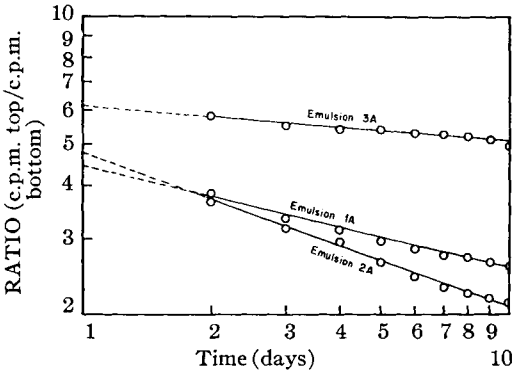


Fig. 2.—Typical log-log creaming curves.

method of least squares (13) was used; and to determine the best linear correlation, the correlation coefficients were calculated. The results of these tests are in Table II. The best linear correlation for emulsions 1A and 1A′, as for the remainder of the emulsions studied, was the log-log correlation. Thus, the mathematical expression was $\ln Y = \ln a + b \ln X$, where Y is the ratio of c.p.m. at the top of the cell to c.p.m. at the bottom of the cell, a is the Y intercept, b is the radioisotopic creaming rate (slope of the line), and X is the time. The general equation would therefore be $Y = aX^b$. From the radioisotopic creaming rates and the day to day variances, it can be seen that the two apparatuses did duplicate the results. The uncolored labeled and uncolored unlabeled controls of emulsion 1 showed no visible signs of phase separation. However, the colored controls showed a change in color intensity on the 10th day (Table II).

Emulsion 2, containing 0.5% electrolyte (sodium iodide), produced a system with more rapid phase separation. The results of this study are shown in Table II as 2A and 2B. The analyses of variance of both emulsions indicated significant day to day changes in the ratio. The Newman-Keuls test (Table II) indicated that emulsion 2B changed for a longer period of time than emulsion 2A. The radioisotopic creaming rates also indicated that emulsion 2, with 0.5% electrolyte, creamed more rapidly than emulsion 1, even though emulsion 2 had 5% more emulsifier. A change in color intensity of the dyed NaI^{131}-labeled control and the unlabeled control emulsions was observed on the 4th day, al-

though no change was observed in the uncolored samples. However, the same basic emulsion labeled with $C_6H_5I^{131}$ started to cream rapidly on the 6th day. This indicated the $C_6H_5I^{131}$ might be causing an acceleration of the creaming rate. To verify this assumption, unlabeled iodobenzene was added to emulsion 2 and again a rapid phase separation was observed.

Emulsion 3 was prepared to verify further the end counting method using NaI^{131} and to find a procedure for incorporating $C_6H_5I^{131}$ without accelerating the rate of creaming (Table I). Because of the rapid phase separation during the first 48 hours, observed in emulsions 1 and 2, emulsion 3 was counted every 2 hours for the first 12 hours, and every 2 hours from the 24th to 36th hour. After the 36th hour they were counted three times a day at 6-hour intervals. The analysis of variance of all three emulsions indicated a significant day to day change in ratio and the Newman-Keuls test showed the first day to be the only one significantly different from all others. In emulsions 3A and 3B there were no visual changes in the labeled or unlabeled controls. However, the labeled colored control of emulsion 3C indicated creaming had started on the first day. The radioisotopic creaming rates (Table II) of emulsions 3A and 3B were very similar, but that of emulsion 3C was greatly increased. From these results it was concluded that the $C_6H_5I^{131}$ added to the oil before emulsification increased the creaming rate, but when added with liquid petrolatum and the emulsifiers to the finished emulsion, had no effect on the emulsion system.

Emulsion 3A-hourly, 3B-hourly, and 3C-hourly are the results of the first 36 hours of counting of emulsions 3A, 3B, and 3C, respectively. As can be seen from Table II, the best linear correlation was again log-log. This indicated the same type of change in the ratios (log-log) from hour to hour or day to day. From these results it is apparent that if two emulsions that cream in a relatively short time are to be compared, an hour to hour isotopic analysis would be preferred.

DISCUSSION

The advantages of the radioisotopic method of evaluation are (*a*) sampling is simplified and subsequent chemical assays are not necessary, (*b*) there is a continuous indication of uniformity and if phase separation occurs, it is readily detected, (*c*) the isotope method approaches the problem of emulsion stability from the most important standpoint of dispersion uniformity, and (*d*) very small amounts of label can be used and thus not affect the emulsion system.

In this study a gamma emitter (I^{131}) was used as the label because it could be readily synthesized into water-soluble or oil-soluble compounds. However, if a beta emitter such as P^{32} were used to label the water phase, it is felt that the sensitivity would be increased due to the greater absorption of the beta particles as the emulsion tended to cream.

Although the end counting method was superior to the side counting method, it is felt that a combination of the two might be advantageous. To use the two methods in the most expedient manner, a precision turn table, that would hold a desired number of cells could be constructed with a Geiger-Müller tube mounted above and another below it. Then as the table turned it would lock a cell into position accurately between the two Geiger-Müller tubes. To side count the cell, another apparatus could be constructed that would slowly move a third Geiger-Müller tube and scan the cell. Thus with only three Geiger-Müller tubes, a scaler, a rate meter, and automatic recorder for the side scanning, a number of emulsions could be evaluated in a short period of time.

The radioisotopic method of evaluating emulsions appears to be applicable to other dispersed systems. By the use of the end counting method, suspensions could be evaluated for rate of sedimentation. The absorption of the label by the solid particles should increase the sensitivity of the apparatus. The radioisotopic method appears to have industrial application in comparing visually and microscopically statistically identical emulsions in a short period of time. Also, once a radioisotopic creaming rate has been determined for a given emulsion system, production samples can be withdrawn, labeled, and rapidly compared to the predetermined standard rate.

CONCLUSIONS

1. A radioisotopic method has been developed for determining the phase uniformity of emulsions and for detecting and expressing ratios of creaming of emulsions that is applicable to w/o and o/w emulsions with either phase being labeled.

2. The radioactivity added to the emulsions had no effect on the particle size or viscosity of the emulsions.

3. For the emulsion system studied, much less time was required for the radioisotopic evaluation than for visual or microscopic evaluations, and great sensitivity was evidenced by the detection of phase separation before it became visible.

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Kinetics and Mechanism of the Salicyl Alcohol-Bisulfite Reaction

By LOUIS C. SCHROETER

Reaction between bisulfite or sulfite ion and salicyl alcohol appears to follow second-order kinetics over a wide (4.0–9.5) pH range. Results suggest two parallel S_N2 mechanisms involving uncharged salicyl alcohol with bisulfite or sulfite ion. The empirical heat of activation for the reaction was found to be 24 Kcal. mole^{-1}. The reaction shows a "normal" frequency factor of 4×10^{11} L. mole^{-1} sec.$^{-1}$ characteristic of many ion-neutral molecule reactions. Entropy of activation was calculated as −7.1 e.u. with a standard state of moles per L.

SALICYL ALCOHOL reacts with bisulfite to yield *o*-hydroxyphenylmethane sulfonate (1); however, the scope of the reaction is not limited to salicyl alcohol as the substrate. The reaction has proved useful for the synthesis of various *ortho*- or *para*-hydroxybenzylsulfonates and, with modifications, for the preparation of similar alkoxybenzylsulfonates (2). Kinetics and mechanism of the salicyl-bisulfite reaction are of interest not only as a model for this unique reaction but also for comparison with results obtained in a study of the reaction of epinephrine with bisulfite (3). The kinetics and mechanism of epinephrine reaction with bisulfite have been studied in some detail. Two distinct mechanisms have been uncovered: an S_N1 reaction which predominates at pH values below 4 and an S_N2 reaction which predominates at pH values over 5. The catecholamine, epinephrine, differs from salicyl alcohol in that it contains an asymmetric secondary alcohol function and exists as a charged ion over the pH range 3–7.

EXPERIMENTAL

Materials and Apparatus.—Salicyl alcohol, Eastman WL, was recrystallized twice from water, m.p. 87°. All other chemicals were of reagent grade. A Beckman GS pH meter, a Cary model 11 recording spectrophotometer, and a Perkin-Elmer model 221 spectrophotometer were used.

Kinetic Studies.—Reactant solutions were prepared to contain equimolar (0.03) concentrations of salicyl alcohol and bisulfite or sulfite in 0.30 *M* buffers. Ionic strength of the solutions was adjusted to predetermined values by addition of appropriate amounts of sodium chloride. Solutions were flushed with nitrogen and filled into hard glass ampuls. Ampuls were sealed under nitrogen, then stored in a thermostat in which the temperature variation was less than 0.1°. Sampling was performed by removing ampuls from the thermostat and quickly chilling in an ice-water mixture. Prior to analysis, ampuls were equilibrated briefly at 25°. Available bisulfite was determined by iodometric titration.

Received March 17, 1961, from the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication June 16, 1961.

Product Isolation.—Reaction between equimolar (0.25) concentrations of salicyl alcohol and bisulfite or sulfite ion was carried out at pH 5 and 8 using previously described procedure (2).

Product Isolated from pH 5 (Bisulfite Ion) Reaction in 67% Yield.

Anal.—Calcd. for $C_7H_7NaO_4S$: C, 40.00; H, 3.36; S, 15.26. Found: C, 40.10; H, 3.48; S, 15.41. Infrared spectrum showed the following bands: —OH, 3,180 cm.$^{-1}$; salt, 2,760, 2,620 cm.$^{-1}$; C=C, 1,615, 1,600, 1,515 cm.$^{-1}$; $-SO_3^-$, 1,380, 1,180, 1,155 cm.$^{-1}$; C—O, 1,225, 1,100, 1,040 cm.$^{-1}$; aromatic substitution, 870, 795, 750 cm.$^{-1}$. Ultraviolet spectrum in ethanol: ϵ 213 = 7,250; ϵ 274 = 2,100.

Product Isolated from pH 8 (Sulfite Ion) Reaction in 48% Yield.

Anal.—Calcd. for $C_7H_7NaO_4S$: C, 40.00; H, 3.36; S, 15.26. Found: C, 39.67; H, 3.36; S, 15.19. Infrared and ultraviolet spectra identical with pH 5 product.

RESULTS AND DISCUSSION

Second-order characteristics of the reaction between equimolar amounts of reactants are shown in Fig. 1 in which reciprocal bisulfite or sulfite concentration is plotted against time. The reaction appears to follow second-order kinetics over a wide range of hydrogen ion concentration. This would seem to indicate that both singly and doubly charged ions of sulfurous acid can participate in the reaction with the uncharged salicyl alcohol. Below pH 9, salicyl alcohol, pKa 9.9 (4), exists mainly as a neutral molecule. The apparent second ionization constant of sulfurous acid, Ka'_2 4.5×10^{-7} (determined in a nitrogen atmosphere at 65° in solution of ionic strength equal to unity), fixes the ratio of bisulfite to sulfite ion for any hydrogen ion concentration: bisulfite ion predominates at pH 4 with very little sulfite ion present while the converse is true at pH 8.5. This treatment does not consider further possible species such as pyrosulfite which can exist in equilibrium with bisulfite (5).

The pH profile of the reaction is shown in Fig. 2 in which the logarithm of the observed second-order rate constant, k, is plotted as a function of pH. The rate constant shows little variation with respect to hydrogen ion concentration between pH 4 and 5.5. The specific rate constant, k_1, at pH 4.5 and 65° under experimental conditions where bisulfite ion is the main species, was found to be 8.9×10^{-5} L. mole^{-1} sec.$^{-1}$. The relative concentration of

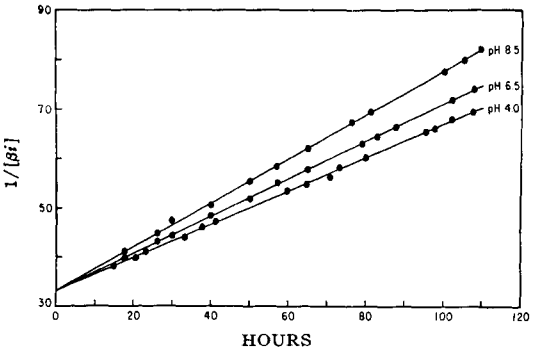


Fig. 1.—Typical second-order plots in which reciprocal bisulfite-sulfite concentration $[\beta i]$ is plotted as a function of time. Equimolar (0.03) solution of recatants in 0.30 M buffer with total ionic strength of 1.0 reacted under nitrogen at 65°. The specific rate constant at pH 4 is 0.32 L. mole^{-1} hr.$^{-1}$ while at pH 8.5 it is 0.45 L. mole^{-1} hr.$^{-1}$.

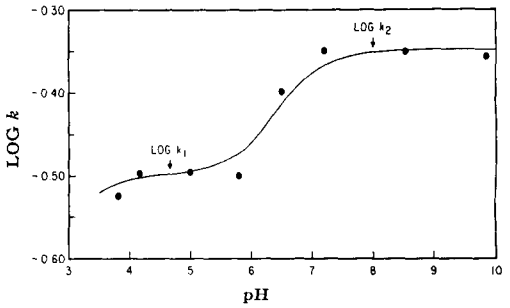
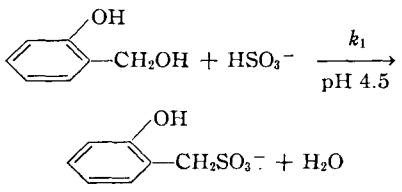


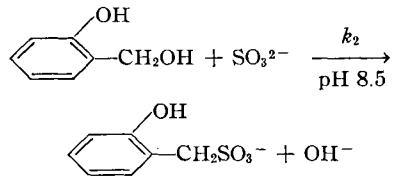
Fig. 2.—Logarithm of second-order rate constants (L. mole^{-1} hr.$^{-1}$) obtained by reacting equimolar (0.03) reactants in various 0.30 M buffers under nitrogen at 65°. Ionic strength of solutions adjusted to 1.0. Theoretical values indicated by solid line calculated from Eq. 5. Experimentally determined values indicated by solid circles. Log $k_1 = -0.50$; log $k_2 = -0.35$.

sulfite species and the observed rate constant increases with pH at pH values above 6 (sulfurous acid pKa'$_2$ 6.35, 65°, μ 1.0). When the added thio compound is totally present as sulfite species (pH > 8), the observed rate constant shows no further pH dependence. The specific rate constant, k_2, at pH 8.5 and 65° under experimental conditions where sulfite ion is the main species, was found to be 1.25×10^{-4} L.$^{-1}$ mole^{-1} sec.$^{-1}$. The results suggest parallel Sn2 reactions which lead to identical hydroxybenzylsulfonates

(A) 

$$\frac{-d(SA)}{dt} = \frac{-d(HSO_3^-)}{dt} = k_1 (SA) (HSO_3^-) \qquad \text{(Eq. 1)}$$

where (SA) = salicyl alcohol concentration.

(B) 

$$\frac{-d(SA)}{dt} = \frac{-d(SO_3^{2-})}{dt} = k_2 (SA) (SO_3^{2-}) \qquad \text{(Eq. 2)}$$

The overall rate expression is the sum of Eqs. 1 and 2

$$\frac{-d(SA)}{dt} = k_1 (SA) (HSO_3^-) + k_2 (SA) (SO_3^{2-}) \qquad \text{(Eq. 3)}$$

Equation 3 may be formulated in terms of added thio compound, St, by introduction of the relationship

$$St = (HSO_3^-) + (SO_3^{2-}) \qquad \text{(Eq. 4)}$$

along with the apparent second ionization constant of sulfurous acid, Ka'$_2$ 4.5×10^{-7}, determined under experimental conditions (65°, μ 1.0):

$$\frac{-d(SA)}{dt} = \frac{-d(St)}{dt} = k_1 (SA) (St) + (SA) (St) \frac{(k_2 - k_1)}{\left[\frac{(H^+)}{Ka_2} + 1\right]} \qquad \text{(Eq. 5)}$$

The theoretical pH profile obtained with Eq. 5 is shown in Fig. 2 as a solid line. The approximate validity of the expression is apparent from the fit of experimentally determined points to the theoretical line.

Mechanism (A) indicates that the bisulfite reaction would proceed with no change in hydrogen ion concentration while mechanism (B) shows the formation of one mole of hydroxide ion for each mole of product. Both reactions were found to give the identical product in good yield and, under conditions corresponding to A, the pH was found to remain nearly constant (pH 5 ± 0.5) while under conditions corresponding to B, stoichiometric production of hydroxide ion was observed, as shown in Table I.

TABLE I.—HYDROXIDE ION PRODUCTION IN THE SALICYL ALCOHOL-SULFITE ION REACTION AT PH 8 UNDER REFLUX CONDITIONS[a]

Minutes	[Sulfite], meq.	[Salicyl Alcohol], mmole	Total OH$^-$ Production,[b] meq.
0	50	50	0
35	33.4	32	16.5
90	22.2	21	27.6
120	19.4	20	30.5
150	16.7	17	43.2
210	13.1	12	37.0
390	8.1	8	42.0

[a] Initial solution composition: 0.05 moles salicyl alcohol, 0.05 moles sodium sulfite in 200 ml. of distilled water. This was reacted in a nitrogen atmosphere under reflux conditions with pH maintained at 8. [b] Determined by continuous titration with hydrochloric acid to maintain pH of reaction mixture at 8.

The effect of ionic strength on the rate constant of the reaction is shown in Fig. 3. Increasing the ionic strength results in a slight decrease in the specific rate constant. In dilute solutions ($\sim 10^{-3}$ M) Debye-Hückel theory (6) leads to an equation of the following form

$$\ln k \simeq \ln k_0 + K\sqrt{\mu}\,(Z \text{ salicyl})\,(Z\ HSO_3^-) \quad \text{(Eq. 6)}$$

which predicts no effect due to ionic strength, μ, inasmuch as the product of the charges, Z, of the reactants equals zero (salicyl alcohol is an uncharged molecule). It should be noted that the experimental ionic strengths are greater than this by several orders of magnitude and as such are not amenable to Debye-Hückel treatment. The changes in the activity coefficient of salicyl alcohol and in the apparent ionization constants of sulfurous acid at higher ionic strengths undoubtedly contribute to the observed slight decrease in the specific rate constant. Equations have been developed to extend Eq. 6 to higher ionic strengths by including a first power ionic strength term (7), but the coefficients cannot be predicted with certainty.

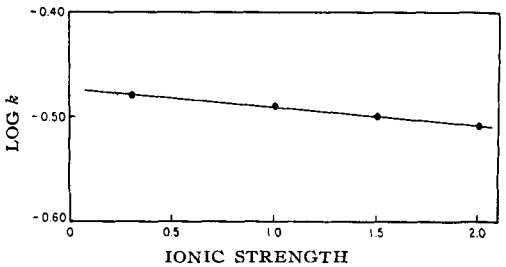


Fig. 3.—Logarithm of second-order rate constant, k_1, (L. mole^{-1} hr.$^{-1}$) as a function of ionic strength. Equimolar (0.03) solution of reactants in 0.30 M acetate, pH 4.9 buffer maintained at 65° under nitrogen atmosphere.

The empirical activation energy, *Ea*, obtained from Fig. 4 is 24 Kcal. mole^{-1}. This corresponds to conditions of mechanism (*A*) where bisulfite ion and neutral salicyl alcohol are reactants. Reaction between epinephrine and bisulfite or sulfite ion exhibits a similar (24 Kcal. mole^{-1}) experimental heat of activation (3) and the analogous Sn2 alkyl halide hydrolytic reactions show similar (23 Kcal. mole^{-1}) activation energies (8). The Arrhenius frequency factor was calculated for the salicyl alcohol-bisulfite reaction using 24 Kcal. mole^{-1} as the empirical activation energy with rate constants obtained at various temperatures. The average value for the frequency factor was found to be 4×10^{11} L. mole^{-1} sec.$^{-1}$; frequency factors for many ion-neutral molecule reactions appear to be of the order 10^{11} L.

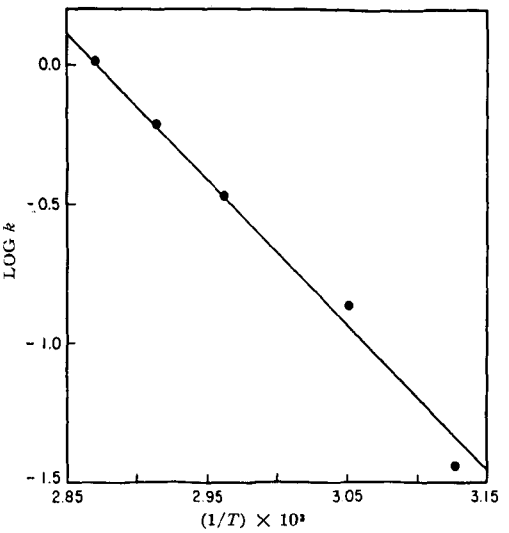


Fig. 4.—Logarithm of second-order rate constant, k_1, (L. mole^{-1} hr.$^{-1}$) as a function of reciprocal absolute temperature. Equimolar (0.03) solution of reactants in 0.30 M acetate, pH 4.9 buffers with total ionic strength of 1.0 reacted under nitrogen atmosphere.

mole^{-1} sec.$^{-1}$ (9) as predicted by collision theory (10). Entropy value for the reaction calculated with the Eyring equation (8)

$$k = \frac{RT}{Nh}\, e^{-\Delta Ha/RT}\, e^{\Delta Sa/R} \quad \text{(Eq. 7)}$$

gave an average value of −7.1 e.u. with a standard state of moles per L. Evaluation of the exponential term, $e^{\Delta Sa/R}$, using the experimental $\Delta Sa = -7.1$ e.u. gave the value of 2.8×10^{-2}; this value appears to be in proper order of magnitude (10^{-2}) for reactions which obey simple collision theory (8).

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Studies on the Mechanism of Action of Phenolic Disinfectants I

Release of Radioactivity from Carbon^{14}-Labeled *Escherichia coli*

By JOSEPH JUDIS

The ability of a number of phenolic disinfectants to cause leakage of cell contents of *Escherichia coli* was studied by exposing cells of the latter that had been labeled by incubating with C^{14} glutamate. Release of cell contents, as indicated by release of radioactivity, was found to be caused by phenol, *p*-chloro-*m*-xylenol, *p*-chloro-*m*-cresol, *p*-chloro-*o*-cresol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and 2,4-dichloro-*m*-xylenol. Similar effects were caused by ethanol, mercuric chloride, acriflavine, and merthiolate. Tween 80 was found to protect *E. coli* from the lethal effects of *p*-chloro-*m*-xylenol and to prevent, in part, leakage of cell contents caused by the latter. Release of radioactivity was directly related to concentration of *p*-chloro-*m*-xylenol and pH had no marked effects.

SINCE THE DISCOVERY of phenol in 1834 and its introduction to antiseptic surgery by Lister in 1867 (1), both phenol and many of its derivatives have become firmly established as germicidal agents. Much of the work with the phenolic disinfectants has centered on practical considerations, such as antimicrobial spectrum, applications, formulations, and the like (2–6). Relatively little work has been done on cytological and biochemical sites of action of these agents. Phenol has long been recognized as a protein precipitant and denaturant and it has been assumed that the latter properties are related to antimicrobial action, especially when applied to vital structures and enzymes of microorganisms (3, 7). More recent work by Gale and Taylor (8), Beckett, Patki, and Robinson (9–11), and others (12–14), but most recently by Joswick and Gerhardt (15) have indicated that the phenolics may exert their lethal activity by damaging the cell membrane or mechanisms which control permeability of the cell membrane. The experiments described below are an attempt to study the ability of various phenol derivatives to cause leakage of cell contents from *Escherichia coli* labeled by incubation with carbon^{14}-tagged glutamate.

METHODS AND MATERIALS

Materials.—All chemicals were of reagent grade and biochemicals were obtained either from the California Corporation for Biochemical Research or Nutritional Biochemicals Corporation. Labeled glutamate (3,4-C^{14}) was obtained from the California Corporation for Biochemical Research and had a specific activity of 2.3 mc. per mmole. The phenol derivatives were a gift from the Ottawa Chemical Company, Toledo, Ohio.

Labeling of Bacteria.—*Escherichia coli* ATCC 11229 was used throughout and was maintained on nutrient agar. Cells for labeling or manometric work were obtained in the synthetic medium (C) of Roberts, *et al.* (16), and had the following composition: ammonium chloride, 2.0 Gm.; disodium phosphate dodecahydrate, 6.0 Gm.; potassium dihydrogen phosphate, 3.0 Gm.; sodium chloride, 3.0 Gm.; magnesium chloride, 0.01 Gm.; sodium sulfate, 0.026 Gm.; glucose, 5.0 Gm.; distilled water, 1 L. The glucose was sterilized separately as a 50% w/v solution and added aseptically at the time of inoculation. The pH of the medium was 6.75. The organism was carried through several transfers on the synthetic medium and a 1% inoculum was used for culture flasks. The latter consisted of 200 ml. of medium in 500-ml. Erlenmeyer flasks and were incubated at 37° on a Burrell wrist action shaker for 24 hours, after which they were harvested by centrifugation. The cells from a single flask were washed once with distilled water and suspended in one-hundredth the original culture volume in saline. The cell suspension was placed in an ice bath, 0.25 ml. of labeled glutamate solution was added (equal to about 230,000 c.p.m.) and allowed to stand for 60 minutes. The cells were then centrifuged, washed three times with cold saline, and suspended in enough saline to give a cell concentration of about 15 to 30 $\times$ 10^9 cells/ml. Generally, about 15 to 20% of the label was taken up by the cells and resulted in an activity of between 100 to 500 c.p.m./10^9 cells. According to the data of Roberts, *et al.* (16), labeling of *E. coli* under these conditions with glutamate results in the disappearance of free glutamic acid and the incorporation of the label of the latter into other, nondiffusable compounds.

Manometric Procedures.—Oxygen uptake was measured in a Warburg respirometer, using the usual techniques (17).

Measurement of Release of Radioactivity.—The reaction mixtures contained, in a final volume of 3.0 ml., about 10 to 20 $\times$ 10^9 cells, phosphate buffer (0.02 *M*, pH 7.2 unless otherwise indicated), phenolic germicide dissolved in 25% v/v ethanol in a maximum volume of 0.5 ml. per reaction mixture,

Received April 28, 1961, from the College of Pharmacy, University of Toledo, Toledo 6, Ohio.
Accepted for publication June 27, 1961.
This work was supported in part by a grant from the University of Toledo Research Foundation.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

and enough saline to bring to volume. When less than 0.5 ml. of germicide solution was used, the difference was made up with 25% v/v ethanol to keep the concentration of the latter constant in the reaction mixtures since ethanol itself caused a release of radioactivity in low concentrations (see below). Enough sodium chloride was added to the phosphate buffers to make them isotonic with saline.

The labeled cells were incubated in the reaction mixtures at room temperature (22 ± 2°) for 10 minutes and removed by centrifugation. The supernatants were plated in 1-ml. quantities on $1^1/_4$ inch aluminum planchets to which 1 to 2 drops of 1% aerosol were added, and allowed to air dry. In cases where the radioactivity of cells was determined, suspensions were transferred to $1^1/_4$ inch aluminum planchets to which had been affixed a disk of lens paper with 1:3 collodion in acetone. Self absorption was corrected for by plating various volumes and calculating correction factors. All assays of radioactivity were carried out in a Nuclear Chicago thin-window gas flow counter and the 0.9 errors were calculated for the values obtained.

Survival in the Presence of Germicides.—The reaction mixtures and cell concentrations were essentially the same as in the experiments involving release of radioactivity and after incubation at 22° for 25 minutes, the viable count at the end of that time was determined by the standard pour plate procedures using nutrient agar as the culture medium.

RESULTS AND DISCUSSION

Of the known antimicrobial agents, the quaternary ammonium compounds and the polypeptide antibiotics are best established as exerting their lethal action by causing damage to the cell membrane (2, 18, 19). Some observations have been made on the ability of phenols to cause release of cellular contents from bacteria (see previously cited references) and the results obtained below would tend to strengthen the view that phenols possess the power to cause leakage from cells.

In Table I, the effects obtained with phenol are shown. At a concentration of phenol which is lethal to *E. coli* in the cell concentration used, the total radioactivity is not released into the supernatant. It also appears that radioactivity is caused to be released by a concentration of phenol which is only slightly bactericidal. Release of radioactivity increases with increase in concentration of phenol. This direct relationship between concentration and leakage has been reported for phenolic compounds (15, 20–22).

TABLE I.—RELEASE OF RADIOACTIVITY FROM LABELED *Escherichia coli* IN THE PRESENCE OF PHENOL[a]

Concentration of Phenol, mg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
0	99.2 ± 8	100
3.3	137.8 ± 9.2	76
6.25	490 ± 16.5	...
12.5	591 ± 18.2	0

[a] The cell concentration was 2 × 10^9 cells/ml. of reaction mixture and had an activity of 550 c.p.m./10^9 cells.

TABLE II.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

Concentration of PCMX, mcg./ml.	Radioactivity Released c.p.m./ml.	Radioactivity Remaining in Cells, c.p.m.	Survival, %
0[a]	150 ± 9.8	1460 ± 37.2	100
66.7	270 ± 12.5	1145 ± 33	...
166.7	431 ± 15.7	710 ± 26.3	32.6
250	591 ± 18.2	676 ± 25.8	...
333.3	685 ± 19.5	702 ± 26.1	0.1

[a] This reaction mixture contained a final concentration of 4.2% ethanol as did all the others containing PCMX for purpose of keeping the PCMX in solution. The cell concentration was 5.2 × 10^9 cells/ml. of reaction mixture and had an activity of 164 c.p.m./10^9 cells.

TABLE III.—EFFECT OF ETHANOL ON THE RELEASE OF RADIOACTIVITY FROM *Escherichia coli*[a]

Concentration of Ethanol, % by Volume	Radioactivity Released, c.p.m./ml.	Survival, %
0	53.9 ± 6.4	100
2.1	131.5 ± 9	...
4.2	251.1 ± 12.2	100
8.33	300.3 ± 13.2	100
12.5	325.5 ± 13.7	58
25.0	392.7 ± 15	0.011

[a] The cell concentration was 4.6 × 10^9 cells per ml. of reaction mixture and had an activity of 252 c.p.m./10^9 cells.

TABLE IV.—EFFECT OF pH ON SURVIVAL AND RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

pH	Concentration of PCMX, mcg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
4.6	0[a]	391 ± 14.9	100
	333.3	1463 ± 28.3	0.1
7.2	0	349 ± 14.1	100
	333.3	1238 ± 26.1	0.19
8.6	0	605 ± 18.4	100
	333.3	1163 ± 25.2	0.02

[a] All reaction mixtures contained a final concentration of 4.2% ethanol. The cell concentration was 4.6 × 10^9/ml. of reaction mixture and had an activity of 471 c.p.m./10^9 cells.

p-Chloro-*m*-xylenol, a halogenated phenol which is a much more potent germicide than phenol, gave similar results (Table II) but was active in much lower concentrations than phenol, both for release of radioactivity and killing.

Because most of the phenol derivatives have a low water solubility, it was convenient to dissolve them in 25% v/v ethanol, resulting in the presence of a certain concentration of ethanol in the reaction mixtures (4.2%). A control was always included which contained the same concentration of ethanol as was present in the reaction mixtures containing phenolics dissolved in ethanol, and these controls always showed greater release of radioactivity than was obtained by incubating labeled cells in the complete absence of ethanol. It seemed of interest to determine whether ethanol itself was capable of causing release of labeled compounds, and in Table III it can be seen that concentrations of ethanol which are not at all lethal do affect the permeability

TABLE V.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF VARIOUS PHENOLIC DISINFECTANTS[a]

Addition	Concentration, mcg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
Nothing	...	96 ± 7.9	100
4.2% Ethanol control	...	432 ± 15.6	100
p-Chloro-*m*-xylenol	166.7		32.6
	333.3	1205.6 ± 25.7	0.1
p-Chloro-*m*-cresol	333.3	911.6 ± 22.5	85
	666.7	1174.4 ± 25.4	0.24
p-Chloro-*o*-cresol	333.3	665.6 ± 19.2	44
	666.7	949 ± 22.9	0.17
2,4-Dichlorophenol	333.3	608 ± 18.2	76
	666.7	1005 ± 23.5	0.14
2,4,6-Trichlorophenol	333.3	709 ± 19.9	68.5
2,4-Dichloro-*m*-xylenol	41.7	745 ± 20.3	60
	83.4		2.72
Phenol	12,500	1149 ± 25.1	0

[a] The cell concentration was 3.8 × 10^9 cells/ml. of reaction mixture and had an activity of 520 c.p.m./10^9 cells.

of the cell membrane of *E. coli*, and this leakage is undoubtedly involved in the bactericidal action of ethanol.

pH has been considered to be a factor affecting the germicidal activity of phenolics, but the general conclusion must be that predictions as to effects are difficult to make and the interaction of a specific germicide, the environment, and the test organism must be individually evaluated (5). For example, with *sec*-amyltricresol, the antibacterial action against *Micrococcus pyogenes* var. *aureus* decreased with increasing pH but increased with increasing pH in the case of *E. coli* (23). The results shown in Table IV indicate that whether one measures release of radioactivity or per cent survival after a given time interval of exposure, pH does not have marked effects on the activity of *p*-chloro-*m*-xylenol under the conditions used.

A number of other phenol derivatives were examined for their ability to cause release of radioactivity from *E. coli*, and the latter effect seems to be caused generally by phenolic germicides, as can be seen in Table V. It is interesting to note that the concentration which causes approximately the same amount of killing also results in the release of about the same proportion of the radioactivity of the cells. The ability to cause release of radioactivity from labeled cells is a property of other antimicrobial substances, as is indicated in Table VI where results with acriflavine, merthiolate, and mercuric chloride are shown.

It has been demonstrated that certain surface-active agents interfere with the antibacterial activity of phenolics (5, 24), especially Tween 80, and it seemed interesting to determine whether Tween 80 under the experimental conditions used above would be capable of preventing the lethal effects of *p*-chloro-*m*-xylenol and also inhibit the release of radioactivity from *E. coli*. Table VII gives typical results. Tween 80, under the experimental conditions, is capable of preventing the lethal effects of *p*-chloro-*m*-xylenol and also inhibited to a large degree, but not completely, the release of radioactivity from the cells. It is interesting to note that while Tween 80 has high surface activity itself, it does not cause release of radioactivity from or significant killing of *E. coli* in a concentration of 1%. Apparently the mode of action of the phenolic germicides involves something beyond simple surface activity. Table VIII shows the ability of Tween 80 to protect *E. coli* from the inhibition of glucose oxidation caused by *p*-chloro-*m*-xylenol. This protective effect seems to be exerted at all three of the pH's at which it was determined.

It should be pointed out that the experiments reported above represent a survey of the ability of phenolic germicides to affect the integrity of the cell membrane of *E. coli* as measured by release of radioactivity from labeled cells, and it is believed that the data presented in addition to those of other workers,

TABLE VI.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF SEVERAL ANTIMICROBIAL SUBSTANCES

Additions[a]	Concentration, mcg./ml.	Radioactivity Released, c.p.m./ml.
Nothing	...	53.9 ± 6.4
4.2% Ethanol control	...	251.1 ± 12.2
p-Chloro-*m*-xylenol	333.3	554 ± 17.7
Acriflavine	2000	560.3 ± 17.4
Merthiolate	1000	502.7 ± 16.8
Mercuric chloride	1000	768 ± 20.7

[a] The mixture containing *p*-chloro-*m*-xylenol contained a final concentration of ethanol of 4.2% and those of the other substances contained no ethanol. The cell concentration was 4.6 × 10^9 cells per ml. of reaction mixture and had an activity of 252 c.p.m./10^9 cells.

TABLE VII.—EFFECT OF TWEEN 80 ON THE RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

Additions[a]	Radioactivity Released, c.p.m./ml.	Survival, %
None	76 ± 7.1	100
4.2% Ethanol control	349 ± 14.1	100
p-Chloro-*m*-xylenol (333.3 mcg./ml.)	1237.5 ± 26.1	0.19
p-Chloro-*m*-xylenol (333.3 mcg./ml.) and Tween 80 (1%)	739 ± 20.2	78
Tween 80 (1%)	88.7 ± 7.6	...
Tween 80 (1%) and 4.2% ethanol	390 ± 14.8	53

[a] The reaction mixtures containing *p*-chloro-*m*-xylenol contained 4.2% ethanol. The cell concentration was 4.6 × 10^9 cells/ml. of reaction mixture and had an activity of 471 c.p.m./10^9 cells.

TABLE VIII.—EFFECT OF pH AND TWEEN 80 ON THE INHIBITION OF GLUCOSE OXIDATION BY *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL[a]

Concentration of *p*-Chloro-*m*-xylenol, mcg./ml.	Q_{O_2}					
	pH 4.3		pH 7.2		pH 8.6	
	Tween 80		Tween 80		Tween 80	
	Absent	Present	Absent	Present	Absent	Present
0	39	45	46.7	46.1	59.8	52.5
83.3	42	43.5			55.9	64.5
166.7	0	40.6	38.9	54.5	9.1	52.5
333.3	0	18.0	19.5	24.7	0	37.5
500	0	...	0	...	0	...
666.7	0	0	0	2.6	0	5.3
833.3	0	0	0	2.6	0	4.5

[a] The flasks contained 140 μM of glucose, phosphate buffer in a final concentration of 0.034 M and of the indicated pH, and 8.5 mg. dry weight of cells in a final volume of 3.0 ml. plus 0.2 ml. of 10% potassium hydroxide in the center well. Tween 80, where used, had a final concentration of 1% and the *p*-chloro-*m*-xylenol was dissolved in 25% ethanol and added in an amount to give the indicated final concentrations. All flasks had a final concentration of 4.2% ethanol (which did not affect oxygen uptake). Temperature was 30°.

do strengthen the hypothesis that the cell membrane is a likely site of action of these germicides. The data also indicate that "leakage" of radioactive cell contents can occur in cell suspensions without a detectable drop in viable count. It would therefore seem that this type of damage to the cell membrane can be tolerated and repaired to a certain degree but, beyond that point, damage is irreparable and death of the cell ensues. The question has been raised as to which comes first, leakage or death (25), and the data would seem to indicate that leakage precedes death. An interpretation of the structural basis for the release of radioactivity from labeled *E. coli* cells must include several possibilities: (*a*) change in permeability of the osmotic barrier of the cell membrane with escape of normal cytoplasmic constituents; (*b*) "uncoupling" of cytoplasmic constituents with subsequent release from the cell; (*c*) a combination of the above. Differentiation of these effects will require experiments involving treatment of labeled cells with substances known to affect the cell membrane without causing uncoupling of cytoplasmic constituents. If phenolics cause release of additional radioactivity from cells so treated, it should be attributable to uncoupling of cytoplasmic constituents. An approach to this type of experiment has been reported by Beckett, Patki, and Robinson (26) and should be extended.

Much of the evidence for the mechanism of action of phenolic germicides indicates that their effect is due to physical damage of the cell membrane. If that is true, and their action is thus relatively nonspecific biochemically, there should be some explanation as to why some strains and species of bacteria are so resistant, relatively. Some possibilities are: (*a*) Resistant strains are incapable of binding the germicide chemically and thus are not damaged since lethal concentrations in the medium would normally be much too low to cause damage to proteins without some sort of concentration, such as at the cell surface or due to absorption within the cell. The inability to bind antibiotics has been observed in bacteria which are resistant to these antibiotics (21). (*b*) The chemical nature of the cell membrane is different in resistant strains so that the chemical site of damage is modified to be resistant. Resistance to quaternary ammonium compounds has been shown to be due to the presence of a high concentration of lipids in the organism which is far above the concentration usually found in sensitive organisms (27, 28). (*c*) Development of mechanisms for detoxication and/or degradation of the phenolic germicide.

It seems unlikely that resistance to phenolic germicides would be due to development of alternate metabolic pathways, since much of the data in the literature would suggest that higher concentrations of germicide are needed for enzyme inhibition than for killing (29–32) and would lead one to the conclusion that inhibition of metabolism is probably not the lethal mechanism. The data in Tables II and VIII seem to agree with this general conclusion, in that a concentration of *p*-chloro-*m*-xylenol of 333.3 mcg./ml. reduced the viable count by 99.9% but inhibited oxidation of glucose to somewhat less than half of normal.

SUMMARY

It has been shown that phenolic disinfectants are capable of causing a release of radioactivity from *E. coli* cells labeled by incubation with carbon^{14}-labeled glutamate. This release of radioactivity is related to concentration of the phenolic compound, occurs with concentrations of the latter not necessarily lethal, and is not markedly affected by pH in the case of *p*-chloro-*m*-xylenol. A number of other phenolic disinfectants, such as *p*-chloro-*m*-cresol, *p*-chloro-*o*-cresol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,4-dichloro-*m*-xylenol, and phenol caused a similar release of radioactivity. Tween 80 at a concentration of 1% can prevent, in part, the release of radioactivity caused by *p*-chloro-*m*-xylenol and protect *E. coli* from otherwise lethal concentrations of the latter. Other substances found to be capable of causing release of radioactivity were ethanol, mercuric chloride, merthiolate, and acriflavine.

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Rheological Properties of Corn Oil Emulsions with Methylcellulose

By B. B. SHETH, DUNCAN E. McVEAN, and ALBERT M. MATTOCKS

Flow properties of a series of emulsions containing methylcellulose 1,500 cps. in eight concentrations and corn oil in 10 concentrations were measured. Known equations for non-Newtonian flow were not satisfactory for these systems, and a new equation, called the "viscoelastic flow equation" was derived and fitted to the data. The equation is $\eta_a = Ae^{-\alpha S} + Be^{-\beta S} + \eta_\infty$, where η_a is apparent viscosity, S is shear rate, η_∞ is ultimate viscosity, and the other terms are constants. From this expression limiting viscosity, η_0 could be obtained as A + B + η_∞. Values of η_0 were then fitted to fluidity plots to obtain intrinsic viscosities. These values agree with Taylor's equation only where the dispersion medium had high viscosity.

ALTHOUGH emulsions may be regarded as suspensions of spherical particles, their rheological properties are more complex than those of suspensions of solid particles. The equation of Einstein governing the viscosity of suspensions, may be expressed as

$$\eta_r = 1 + kC_v \qquad \text{(Eq. 1)}$$

where η_r is relative viscosity at low rates of shear, C_v is concentration by volume of suspended particles, and k is the Einstein constant or intrinsic viscosity, equal to 2.5 for spherical particles. This was modified for emulsions by Taylor (1), who took into account the flow induced in the suspended droplets. From hydrodynamic considerations Taylor derived the following

$$\eta_r = 1 + kC_v\left[\frac{\eta_1 + 0.4\eta_2}{\eta_1 + \eta_2}\right] \qquad \text{(Eq. 2)}$$

where η_1 is the limiting viscosity of the oil in the droplet, $\eta_{0\,(oil)}$, and η_2 is that of the solvent, $\eta_{0(solv.)}$. In a later work, Taylor (2) pointed out that deformation of fluid particles might occur during shear to form ellipsoids, and Oldroyd (3) showed that interfacial tension at the particle surface may retard flow within the particle. Other complexities which have been noted are the formation of adsorbed films with viscosities different from that of the medium or the droplet (3, 5).

In spite of these complicating factors, Nawab and Mason (6) found good agreement with Taylor's equation with one set of carefully prepared dilute emulsions, though most series did not agree, as has been the common result of other workers. In a most interesting fashion, Mason and Bartok (4) exhibited flow patterns and particle deformation of droplets in suspension with a rheometer which allowed the particles to be photographed in shear.

One of the difficulties encountered in the testing of the basic equations of Einstein and Taylor has

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been the limitation that Eq. 1 is applicable only to infinitely dilute suspensions, and various modified equations have been used to extrapolate flow data of suspensions of finite concentrations and thus obtain values representing Einstein conditions. The Einstein equation, modified to allow for hydrodynamic interactions occurring at finite concentration may be expressed as

$$\eta_r = 1 + 2.5\ C_v + k_1 C_v^2 + \ldots \quad \text{(Eq. 3)}$$

Some workers have applied this equation directly as a polynomial, others have utilized a curve of η_{sp}/C_v *vs.* C_v, where $\eta_{sp} = \eta_r - 1$, and extrapolated to $C_v = 0$ to obtain

$$\lim_{C_v \to 0} \eta_{sp}/C_v$$

as the Einstein constant. Other relationships, such as logarithmic functions, have been used with limited success. Recently, Ford (7) noted that if relative fluidity, ϕ_r, were used, plots of ϕ_r *vs.* C_v were usually linear up to rather high concentrations, as had been observed much earlier by Bingham (8), and that the fluidity relationship was a more suitable method for evaluating the Einstein constant. The equation was expressed as

$$\phi_r = 1 - 2.5C_v + \ldots \quad \text{(Eq. 4)}$$

which is the reciprocal of the Einstein equation in the form

$$\eta_r = 1 + 2.5C_v + 6.25C_v^2 + \ldots \quad \text{(Eq. 5)}$$

The report by Ford has thus supplied a more reliable method for testing the basic flow equations with suspensions of finite concentration.

Pharmaceutical emulsions are usually non-Newtonian in nature, since they generally contain thickening agents such as natural gums and synthetic cellulose deratives which are, themselves, non-Newtonian. This fact makes it more difficult to apply the basic equations governing flow to such emulsions because the apparent viscosity measured will be variable, depending on the rate of shear of measurement. The difficulty may be overcome, however, if flow equations which will accurately predict the limiting viscosity, η_0, or viscosity at infinitely low shear rate, are available. Since the sedimentation of particles in emulsions is thought to occur at extremely low shear rates and since the flow characteristics at low shear rates show promise of furnishing a means for evaluating interfacial tension (6) and occurrence of aggregation (4), the objective of obtaining suitable flow equations is of importance.

An equation for describing non-Newtonian flow may be evaluated by three criteria: (*a*) its agreement with observed data, (*b*) its accuracy in furnishing correct values of η_0, and (*c*) its theoretical foundation.

Few theoretical equations have been derived for non-Newtonian flow, the exceptions being that of Ree and Eyring (9) involving an inverse hyperbolic sine which is difficult to evaluate, and that of Williamson (10) which was used empirically for some time before being derived theoretically (11).

The equation of Williamson has been shown to fit a wide variety of non-Newtonian flow curves (12–14), but it was also noted that the asymptote to the curve was unrealistic, and a new equation, called the "structure equation," was utilized which gave more accurate values of the asymptote at high shear rates (14, 15). Unfortunately, the structure equation could not be fitted to most flow curves without the assumption of a fixed yield value which often does not agree with the observed data. The concept of a yield value representing a nonyielding condition which suddenly disrupts at a fixed stress is also not in agreement with the recent findings of McVean (16), who measured the strain of gels to find them more aptly described by a viscoelastic equation

$$F = Ae^{-\alpha t} + Be^{-\beta t} \quad \text{(Eq. 6)}$$

where F is stress, A, α, B, β, are constants, and t is time at constant strain. A non-Newtonian flow equation utilizing this viscoelastic concept of yield value which has been devised and used in these laboratories is as follows

$$\eta_a = F/S = Ae^{-\alpha S} + Be^{-\beta S} + \eta_\infty \quad \text{(Eq. 7)}$$

where η_a is the apparent viscosity at a given shear, S, and η_∞ is the ultimate high shear viscosity. This equation has been found to fit a variety of experimental flow curves quite accurately without the assumption of a fixed yield value. Equations of this type have been used for viscoelastic bodies, being represented by models containing two Maxwell elements in parallel (17). It might be expected that more or fewer exponential terms may be required for a given system, since the number will depend on the breadth of distribution of relaxation times of the non-Newtonian components.

The work reported in this paper illustrates the application of Eq. 7, called the "viscoelastic flow equation," to a series of emulsions.

EXPERIMENTAL

Preparation of Emulsions.—A sol was prepared by adding methylcellulose 1,500 cps., with stirring, to about one-half the calculated amount of water, previously heated to 70°. A preservative consisting of 0.1% of a mixture of three parts methylparaben

and one part propylparaben was dissolved in the hot water before incorporation of the methylcellulose. The mixture was made up to volume with cold water and stirred with chilling until its temperature was 0–5°; it was then stored in a refrigerator for at least 2 days before being used. The concentration of methylcellulose was accurately determined by drying a weighed sample of the final sol.

A basic corn oil emulsion was prepared with 60% corn oil (η = 0.4449), 5% of a mixture of three parts of Span 80 and one part of Tween 80, and 35% water. The emulsion was homogenized in a Manton-Gaulin homogenizer (model 15M8BA) at 3000 lb./in.^{2}. Droplet size of the dispersed phase was found to be quite uniform, 90% of the particles being in the range of 0.5–3 μ, and the size varied little with variation in homogenization pressure or with dilution with varying amounts of methylcellulose sol or water. Emulsions containing varying concentrations of oil and methylcellulose were obtained by dilution of the basic emulsion with water and methylcellulose sol, so that only one lot of basic emulsion and sol were used throughout the study. The final emulsions were stirred gently to remove air and allowed to stand at room temperature for 3 days before measurement.

Measurement of Flow Properties.—The emulsions were placed in an automatic recording viscometer with rotating cup and fixed bob (18). Shear rates were varied from 129 to 2,500 sec.$^{-1}$, and from the recorded curves data points representing 19 equally spaced intervals of shear were recorded for computation.

Computation of Flow Constants.—From the shear-stress data, values of η_a were calculated and plotted *vs.* S on 12 × 18 inch graph paper. These were placed on an X-Y recorder and fitted to the viscoelastic flow equation by means of an analog computer. Values of the constants were read directly from the computer.

Results.—Since the original stress-shear data are quite extensive and would require excess space, they are not reproduced in this paper. They are available, however, in the original thesis (18). The constants of the viscoelastic flow equation are presented in Table I. The constants of the equation were used to compute limiting viscosities, η_0, by the following relationship

$$\eta_0 = \lim_{S \to 0} \eta_a = A + B + \eta_\infty$$

These, in turn, were used to calculate values of ϕ_r, which were plotted *vs.* C_v to obtain slopes comparable to Einstein's constant. Constants of the viscoelastic flow equation and values of ϕ_r are presented in Table I, and slopes, or intrinsic viscosities, are given in Table II, along with values calculated from Taylor's equation. A typical curve fitted on the computer is shown in Fig. 1, a typical fluidity plot is given in Fig. 2, and the relationship between values of k and η_2 are presented in Fig. 3.

DISCUSSION

It was found that the viscoelastic flow equation fitted all the flow curves of this series of emulsions quite well. The fitting of five constants, however,

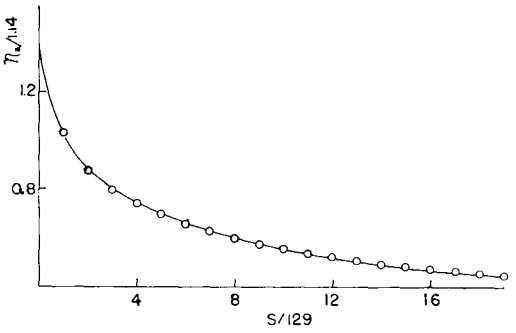


Fig. 1.—Typical flow curve for emulsion. Oil, 16.2%, methylcellulose 1.170%. Circles represent experimental points; line is computer-drawn.

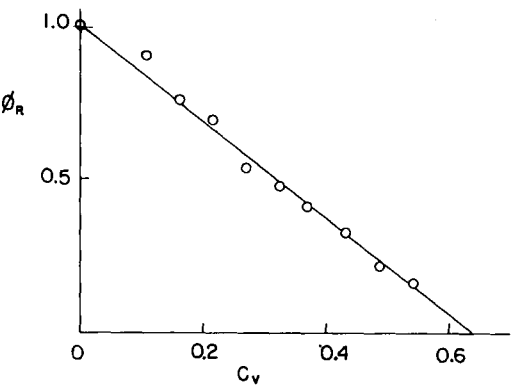


Fig. 2.—Typical fluidity plot, for 0.651% methylcellulose as continuous medium.

TABLE I.—CONSTANTS OF THE VISCOELASTIC FLOW EQUATION AND RELATIVE FLUIDITIES

Methylcellulose, % w/v	Corn Oil, % v/v	A Poises	B Poises	η_∞ Poises	$\alpha \times 10^2$ sec.	$\beta \times 10^2$ sec.	ϕr
0.326	0.0	0.0094	0.0440	0.0440	0.798	0.593	1.0000
	10.8	0.0212	0.0418	0.0728	1.426	0.655	0.7172
	16.2	0.0183	0.0533	0.0655	1.112	0.880	0.7104
	21.6	0.0185	0.0755	0.0749	0.977	1.019	0.5767
	27.0	0.0309	0.0819	0.0898	1.159	0.922	0.4808
	32.4	0.0278	0.0910	0.0949	1.225	0.915	0.4558
	37.9	0.0593	0.1191	0.1399	1.225	1.070	0.3060
	43.3	0.0772	0.1789	0.1416	1.151	1.070	0.2449
	48.7	0.1463	0.2497	0.1923	1.178	1.070	0.1656
	54.2	0.2684	0.3768	0.2474	1.426	0.911	0.1091

TABLE I (*continued*)

Methyl-cellulose, % w/v	Corn Oil, % v/v	A Poises	B Poises	η_∞ Poises	$\alpha \times 10^3$ sec.	$\beta \times 10^2$ sec.	ϕr
0.434	0.0	0.0168	0.0600	0.0710	0.837	0.601	1.0000
	10.8	0.0251	0.0688	0.0872	0.709	0.702	0.8161
	16.2	0.0359	0.0593	0.0948	0.806	1.194	0.7779
	21.6	0.0318	0.0497	0.1131	0.961	0.674	0.7595
	27.0	0.0583	0.0963	0.1146	0.953	0.880	0.5490
	32.4	0.0742	0.0745	0.1468	0.953	1.039	0.5002
	37.9	0.1002	0.1248	0.1723	0.953	0.845	0.3720
	43.3	0.1262	0.1318	0.1968	0.829	0.465	0.3250
	48.7	0.1724	0.3095	0.2376	0.860	0.640	0.2054
	54.2	0.3795	0.4933	0.2994	1.229	0.829	0.1261
0.543	0.0	0.0405	0.0510	0.1015	1.050	0.671	1.0000
	10.8	0.0459	0.0436	0.1163	0.791	0.957	0.9378
	16.2	0.0488	0.0491	0.1307	0.891	0.911	0.8443
	21.6	0.0702	0.0834	0.1458	0.783	0.911	0.6446
	27.0	0.0718	0.0898	0.1610	0.597	0.481	0.5983
	32.4	0.0944	0.0997	0.1829	0.705	0.512	0.5119
	37.9	0.1380	0.1895	0.2144	0.950	0.814	0.3562
	43.3	0.1966	0.2439	0.2345	0.853	0.814	0.2859
	48.7	0.2954	0.3337	0.2890	1.046	0.744	0.2102
	54.2	0.3787	0.7670	0.3418	0.950	0.667	0.1297
0.651	0.0	0.0872	0.0669	0.1285	0.558	1.209	1.0000
	10.8	0.0974	0.0559	0.1606	0.616	0.450	0.9003
	16.2	0.1238	0.0541	0.1865	0.833	0.864	0.7548
	21.6	0.1476	0.0673	0.1944	0.833	0.841	0.6904
	27.0	0.1670	0.1443	0.2190	0.903	0.880	0.5329
	32.4	0.2028	0.1596	0.2309	0.717	0.791	0.4763
	37.9	0.2281	0.1971	0.2666	0.740	0.547	0.4085
	43.3	0.3079	0.2619	0.3014	0.764	0.574	0.3244
	48.7	0.3981	0.5849	0.3396	0.702	0.562	0.2137
	54.2	0.5887	0.7943	0.3868	0.833	0.655	0.1597
0.780	0.0	0.1286	0.0664	0.1735	0.767	0.659	1.0000
	10.8	0.1448	0.0917	0.1886	0.473	0.244	0.8669
	16.2	0.1703	0.0258	0.2359	0.853	0.481	0.8530
	21.6	0.1899	0.1093	0.2302	0.853	0.721	0.6961
	27.0	0.2323	0.1052	0.2653	0.795	0.430	0.6113
	32.4	0.2796	0.1864	0.2940	0.798	0.574	0.4849
	37.9	0.4643	0.3497	0.3825	0.981	0.705	0.3080
	43.3	0.4496	0.5510	0.3804	0.981	0.578	0.2668
	48.7	0.5516	0.7150	0.4485	0.981	0.721	0.2149
	54.2	0.6360	1.2299	0.4625	0.771	0.942	0.1583
1.002	0.0	0.2387	0.1789	0.2711	0.593	0.198	1.0000
	10.8	0.3690	0.3093	0.3203	0.886	1.260	0.6897
	16.2	0.3668	0.3832	0.3403	0.886	0.578	0.6317
	21.6	0.3905	0.3394	0.3832	0.822	0.453	0.6187
	27.0	0.4854	0.2609	0.4270	0.961	0.453	0.5870
	32.4	0.5597	0.5044	0.3805	0.671	0.628	0.4767
	37.9	0.5475	0.5091	0.4750	0.798	0.388	0.4497
	43.3	0.6922	0.7071	0.5063	0.860	0.465	0.3614
	48.7	0.8183	1.0831	0.5746	0.919	0.484	0.2782
	54.2	0.9837	1.5268	0.6427	0.922	0.461	0.2184
1.170	0.0	0.5352	0.3303	0.3325	0.674	0.578	1.0000
	10.8	0.6577	0.3471	0.4248	0.961	0.899	0.8380
	16.2	0.6783	0.5412	0.4659	1.074	1.101	0.7108
	21.6	0.6787	0.5557	0.4965	0.934	0.694	0.6921
	27.0	0.7837	0.5262	0.5019	0.895	0.655	0.6612
	32.4	0.8929	0.7083	0.5576	0.969	0.752	0.5549
	37.9	0.9305	1.0850	0.5726	0.860	0.643	0.4629
	43.3	1.0732	1.3143	0.6327	0.891	0.578	0.3967
	48.7	1.2111	1.6743	0.6502	0.891	0.597	0.3388
	54.2	1.4789	2.4507	0.7586	1.027	0.640	0.2555
1.337	0.0	0.9380	0.3622	0.5324	1.085	0.647	1.0000
	10.8	0.8580	0.7305	0.5178	0.756	0.469	0.8701
	16.2	0.8610	0.7329	0.5422	0.760	0.469	0.8579
	27.0	1.2201	0.8764	0.6702	0.973	0.554	0.6624
	32.4	1.4200	1.1021	0.7121	1.089	0.729	0.5666
	37.9	1.4793	1.5896	0.7630	1.050	0.694	0.4782
	43.3	1.5302	1.8524	0.7630	0.946	0.578	0.4421
	48.7	1.6955	2.6111	0.7960	0.953	0.601	0.3592
	54.2	1.5514	3.1197	0.8478	0.903	0.461	0.3304

TABLE II.—RELATIONSHIP OF INTRINSIC VISCOSITY OF EMULSION, k, TO VISCOSITY OF CONTINUOUS PHASE, η_2

η_2, Poises	Found, k	k Calcd. from Taylor's Equation
0.0974	1.699	2.231
0.1478	1.585	2.126
0.1930	1.587	2.046
0.2826	1.572	1.917
0.3685	1.595	1.821
0.6887	1.527	1.589
1.1980	1.384	1.406
1.8326	1.283	1.280

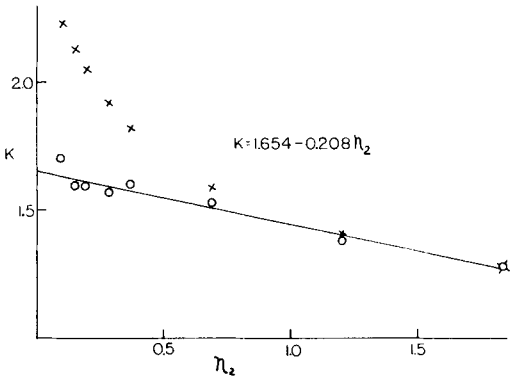


Fig. 3.—Relationship between k and η_2. Circles represent experimental points, X's are values calculated from Taylor's equation. The least squares equation for the line is given.

allows errors in one to be corrected for by errors in another, so that a given constant might not vary in a regular fashion with increases in concentration of emulsion. Nevertheless, the complete equation describes the flow curve quite accurately and the combined constants, such as η_0, do vary in an orderly manner. One might question whether a single value of α and β would suffice for a complete series involving a given suspending agent, since most of the non-Newtonian character might be ascribed to this component. This was attempted, using an approximate average value for α and β, but the errors were increased considerably by this procedure, while approximately the same values of intrinsic viscosity were obtained. It is obvious that accuracy of evaluation of the constants can be improved by extending the range of shear of the measurements.

Values of ϕ_r were invariably linear with respect to C_v, which supports the concept of Ford that fluidities are the terms of choice in evaluating intrinsic viscosity. These findings also favor the use of the viscoelastic flow equation for obtaining low shear viscosities.

From Fig. 3 it is seen that a linear relationship appears to exist between intrinsic viscosity and viscosity of the suspending medium. This is not in agreement with Taylor's equation which predicts nonlinearity throughout the range of η_2. The discrepancy could not be attributed to the formation of a hydrated sheath to give the particle a hydrodynamic volume above the apparent volume, since in such case the constants would be greater than 2.5. Neither could this be attributed to an apparently consistent decrease in viscosity of one of the components, since such a prediction would still call for the nonlinear portion described by Taylor's equation. Doublet formation or aggregation would yield high values for k. Thus, by elimination, the failure to agree with Taylor's equation at low values of η_2 is perhaps best explained by slippage of an adsorbed layer at the surface of the particles, as suggested by Oldroyd (3). It does seem reasonable that the degree of slippage might decrease with increase in η_2, since the layer involved would most likely be of a modified stratum of the medium itself.

These results indicate that the viscoelastic flow equation is of value in describing non-Newtonian flow data. It has the advantage of fitting the observed flow curves quite well without requiring a rigid yield value. It seems likely to have wide applicability by modification of the number of exponential terms, and extrapolated values of limiting viscosity appear to be useful in investigating basic relationships.

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Preparation and Evaluation of Thiamine N-Cyclohexylsulfamate Hydrochloride

By JAMES A. CAMPBELL

The presently available salts of thiamine are known to have a characteristic bitter taste. The N-cyclohexylsulfamic acid salt of thiamine hydrochloride was prepared and was found to have a taste which was greatly improved over that of the presently available salts of thiamine. After characterization, the salt was placed under short term accelerated aging conditions. The data obtained appears to indicate that the salt possesses good stability.

THE THIAMINE MOLECULE contains two basic nitrogen atoms, the quaternary nitrogen of the thiazole ring and the nitrogen of the primary amino group attached to the pyrimidine ring. Two types of thiamine salts are, therefore, possible. The first type involves only the nitrogen of the thiazole ring which is a more strongly basic center than the primary amino function (1). The second type of salt results when further salt formation occurs at the primary amino group. An example of the former would be thiamine mononitrate, whereas an example of the latter would be thiamine chloride hydrochloride.

Thiamine chloride hydrochloride and thiamine mononitrate appear to be the salts of choice in pharmaceutical dosage forms at the present time. The mononitrate is reported to be preferred in dry dosage forms because of greater stability in the dry state (1).

Various salts of thiamine have been reported. Okumura and Sakurai (2, 3), with the aid of ion exchange resins, prepared the following salts: nitrate·HNO_3; acid sulfate; iodide·HI; bromide·HBr; thiocyanate·HCNS; chloride; bromide; iodide; and thiocyanate. Water-soluble disulfonates of thiamine have also been reported by the same authors (4).

Thiamine salicylate (5) was prepared in Germany a number of years ago and was reported to be useful in the treatment of rheumatic and arthritic disease.

Several phosphoric acid derivatives of thiamine are quite well known. These include thiamine phosphoric acid ester chloride, thiamine phosphoric acid ester phosphate salt, thiamine phosphoric acid salt, thiamine triphosphoric acid ester, thiamine triphosphoric acid salt, and others (6).

Although many thiamine derivatives have been described, nothing has been reported concerning the improvement of the bitter taste which is characteristic of thiamine. The primary objective in preparing thiamine N-cyclohexylsulfamate hydrochloride was to obtain a salt of thiamine with improved taste and with stability at least as good as that of the presently available salts of thiamine.

Silver nitrate was reacted with N-cyclohexylsulfamic acid to form insoluble silver N-cyclohexylsulfamate. The silver N-cyclohexylsulfamate was reacted with thiamine chloride hydrochloride to produce thiamine N-cyclohexylsulfamate hydrochloride, leaving behind the insoluble silver chloride. The reactions are illustrated by the equations.

TABLE I.—STABILITY OF B_1 IN MULTIPLE VITAMIN SOLUTIONS UNDER VARIOUS CONDITIONS OF TEMPERATURE AND pH

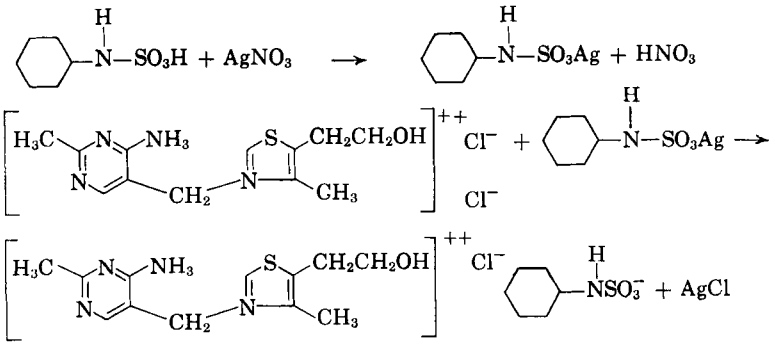
Type of B_1 Salt	Conditions of Storage pH	°C.	Original Assay, mg./ml.	3 Months Assay, mg./ml.	% Loss
TM[a]	3	25	9.99	9.98	0.1
TN-C[b]	3	25	9.90	9.82	1.6
TM	4	25	9.94	9.60	3.4
TN-C	4	25	10.24	10.24	0.0
TM	5	25	9.97	9.26	7.1
TN-C	5	25	9.95	9.40	3.5
TM	3	40	9.99	9.18	8.0
TN-C	3	40	9.90	9.14	7.7
TM	4	40	9.94	7.49	24.7
TN-C	4	40	10.24	7.48	26.9
TM	5	40	9.97	6.43	35.5
TN-C	5	40	9.95	6.69	32.8
TM	3	50	9.99	8.74	12.5
TN-C	3	50	9.90	8.92	9.9
TM	4	50	9.94	5.13	48.3
TN-C	4	50	10.24	5.23	48.0
TM	5	50	9.97	3.38	66.0
TN-C	5	50	9.95	3.45	65.3

[a] Thiamine mononitrate. [b] Thiamine N-cyclohexylsulfamate hydrochloride.

TABLE II.—STABILITY OF THIAMINE N-CYCLOHEXYLSULFAMATE HCl IN MULTIPLE VITAMIN TABLETS

Condition, °C.	Original, mg./Tablet	One Month, mg./Tablet	Three Months, mg./Tablet	% Loss
25	10.1	10.0	10.0	1
40	10.1	9.5	9.8	3
50	10.1	9.1	9.2	9

Received April 28, 1961, from the Pharmacy Research Laboratory, Miles Laboratories, Inc., Elkhart, Ind.
Accepted for publication June 27, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.



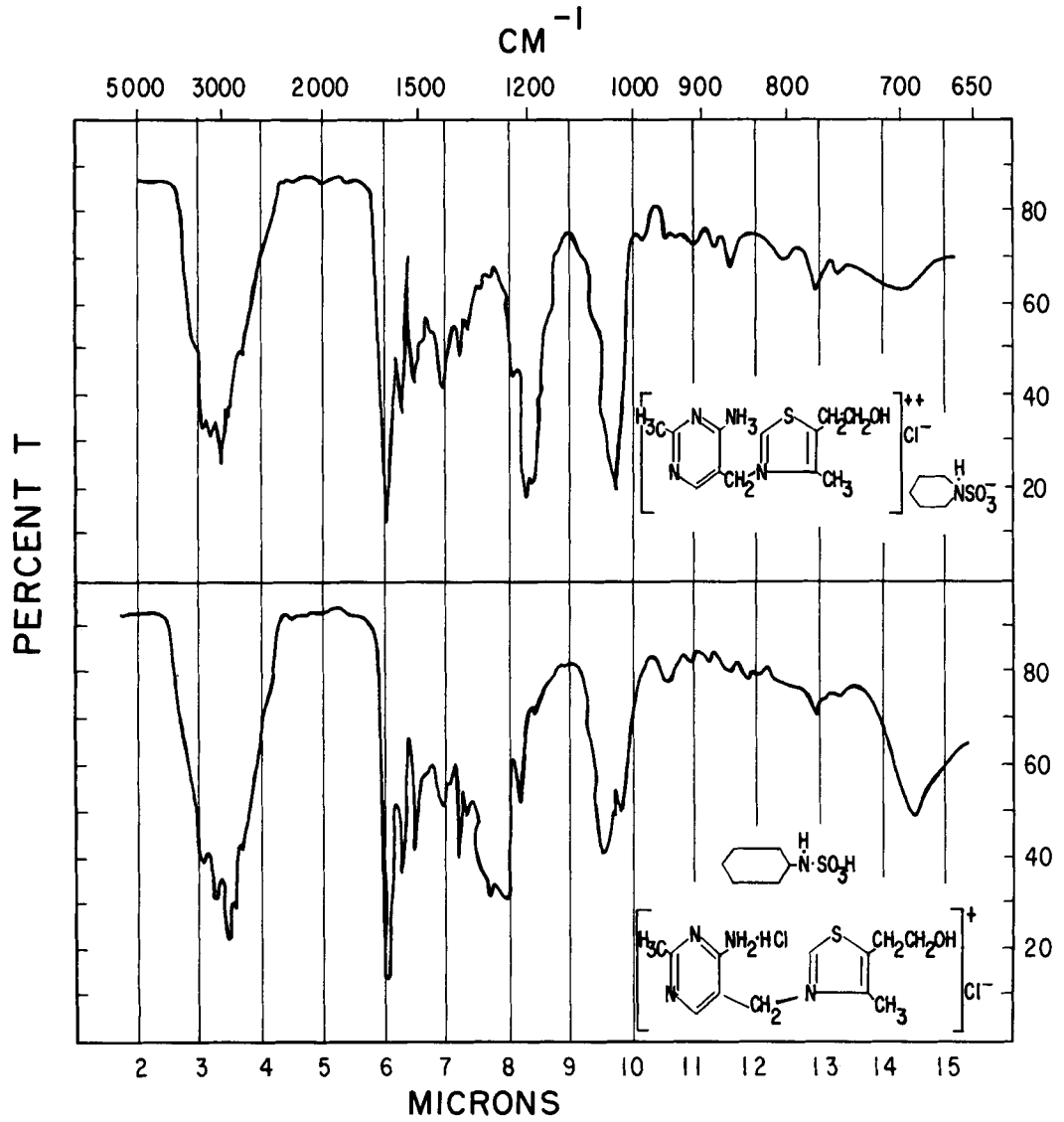


Fig. 1.—Infrared spectra. Above: thiamine N-cyclohexylsulfamate hydrochloride; below: equimolar mixture of thiamine hydrochloride and N-cyclohexylsulfamic acid.

EXPERIMENTAL

Preparation.—A solution consisting of 5 Gm. of silver nitrate in 3 ml. of water was added, with stirring, to a solution consisting of 5.25 Gm. of N-cyclohexylsulfamic acid in 50 ml. of water. The silver N-cyclohexylsulfamate which precipitated was collected on a filter, washed with water, and dried in an oven at 50°.

A 5-Gm. portion of thiamine chloride hydrochloride was dissolved in 10 ml. of water and to this was added 5 Gm. of the silver N-cyclohexylsulfamate. The suspension was stirred at 50° for 30 minutes and was then cooled and filtered. The filtrate was evaporated to dryness under reduced pressure. The solid residue was dissolved in warm methanol; and after cooling, ether was added to the solution to precipitate the final product as a white granular substance having a sweet taste and being freely soluble in water. The salt turned pink at 160° and decomposed between 185 and 190°.

Infrared Spectra.—A potassium bromide pellet was made containing an equimolar mixture of thiamine chloride hydrochloride and N-cyclohexylsulfamic acid. An equivalent concentration of the thiamine N-cyclohexylsulfamate hydrochloride was also incorporated into a potassium bromide pellet and the infrared spectra of both were obtained. The spectra, as shown in Fig. 1, are considerably different. Certain absorption peaks appeared in the spectrum of the compound that were absent in the spectrum of the mixture. These peaks can be observed at 805, 875, 1040, 1170–1220, and 1280–1320 cm.$^{-1}$. Likewise, certain absorption peaks appeared in the spectrum of the mixture that were absent in the spectrum of the compound. These peaks can be observed at 695, 833, 945, and 1065 cm.$^{-1}$.

Anal.—Calcd. for $C_{18}H_{30}ClN_5O_4S_2$: N (total), 14.60; N (basic), 5.83; HCl, 7.60; mol. wt., 480.05. Found: N (total, Kjeldahl), 14.58; N (basic), 6.09; HCl, 7.74.

Stability Studies.—An accelerated aging program was designed and carried out to determine the stability of the compound when combined in multiple vitamin preparations.

Solution Stability.—Three solutions of the following content were prepared

Substance	mg./ml.
Thiamine N-cyclohexylsulfamate HCl	10
Riboflavin-5-phosphate sodium	2
Nicotinamide	50
Panthenol (*dl*)	10
Pyridoxine HCl	5
Ascorbic acid	25
Chlorobutanol	0.6
Water *q.s.*	

Three solutions were prepared identical to that above except that thiamine mononitrate was substituted for the thiamine N-cyclohexylsulfamate hydrochloride in the same concentration.

One solution of each type was adjusted to pH 5 using sodium hydroxide, one solution of each type was adjusted to pH 4 using hydrochloric acid, and one solution of each type was adjusted to pH 3 using hydrochloric acid. Each of the final solutions was assayed for thiamine content and each was subdivided into three portions. The solutions were placed in amber glass bottles and a portion of each solution was stored at each of the following temperatures: 25, 40, and 50°. The solutions were assayed for thiamine content at the end of 3 months. The thiochrome method of assay was used in all assays and the assay results are shown in Table I.

Tablet Stability.—The thiamine N-cyclohexylsulfamate hydrochloride was incorporated into multiple vitamin tablets containing the following vitamins

Vitamins	Amount
Thiamine N-cyclohexylsulfamate HCl	10.0 mg.
Niacinamide	20.0 mg.
Riboflavin	2.5 mg.
Pyridoxine HCl	1.0 mg.
Sodium ascorbate	62.0 mg.
Vitamin B_{12}	1.0 mcg.
Vitamin A	5000.0 units
Vitamin D	500.0 units
Excipients *q.s.*	

The tablets were stored in tightly closed bottles for a period of three months at 25, 40, and 50°. The thiochrome method of assay was performed for thiamine content at zero, 1 month, and 3 month time intervals. The results are shown in Table II.

SUMMARY AND CONCLUSIONS

1. The N-cyclohexylsulfamic acid salt of thiamine was prepared and characterized.

2. The salt was found to have a pleasant taste which was judged to be superior to that of thiamine hydrochloride or thiamine mononitrate.

3. When combined with other vitamins in typical multiple vitamin preparations and subjected to accelerated aging, the salt has demonstrated a stability that compares favorably with that of thiamine mononitrate. It also appears that the optimum pH for the salt in aqueous solution is 3 or below.

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Synthesis of 2-Thiophene Derivatives

By FRED C. ROGERS† and W. LEWIS NOBLES

The objective of this present study is the synthesis of certain new 2-thiophene derivatives to be evaluated as potential antispasmodic, analgesic, or local anesthetic agents. The first part of this study concerns the synthesis of β-aminoketones (Mannich bases). The second segment concerns the synthesis of γ-amino secondary alcohols by chemical reduction of the corresponding β-aminoketones. The third portion describes the synthesis of a series of γ-amino tertiary alcohols by application of the Grignard reaction to the corresponding Mannich bases. The last section of the study involves the preparation of γ-aminoalkyl esters from the corresponding tertiary alcohols.

THE INTEREST in compounds containing thiophene rings as biologically active agents stems primarily from the similar physical and chemical properties of benzene and thiophene. The concept of preparing thiophene analogs of biologically active compounds has stimulated workers in medicinal chemistry to the point that by now it appears that at least one thiophene analog has been prepared for every important therapeutic group containing a benzene nucleus.

The physical and chemical characteristics of thiophene and benzene, as well as some of the biological activities of compounds of this type, can be reasonably well correlated by the application of the concept of isosterism as introduced by Langmuir (1) in 1919 and subsequently developed by Erlenmeyer (2) and others (3, 4). In the case of thiophene, the lower resonance energy and the presence of a hetero atom in the ring, along with the fact that a dipole moment exists, would logically lead one to postulate that thiophene compounds would be more easily degraded and detoxified in the human body. It is generally accepted from experimental evidence, however, that thiophene compounds are more toxic than their benzene analogs. There are important exceptions, however, and it is this realization that inspires further investigations in this field of endeavor.

The preparation of β-aminoketones requires the use of the Mannich reaction (5), which consists of the condensation of formaldehyde with ammonia, or a primary or secondary amine and a compound containing at least one active hydrogen atom. The amine is generally used in the form of its hydrochloride salt. An example of the reaction follows

$$C_6H_5COCH_3 + CH_2O + R_2NH.HCl \rightarrow C_6H_5COCH_2CH_2NR_2 \cdot HCl + H_2O$$

Mannich and Lammering (6) prepared a series of β-aminoketones and found that β-N-piperidinoethyl phenyl ketone, prepared from acetophenone, piperidine hydrochloride, and paraformaldehyde, possessed local anesthetic activity. Levvy and Nisbet (7) prepared a series of β-aminoketones from 2-acetylthiophene and found that 2-thienyl-β-piperidinoethyl ketone hydrochloride and the β-dimethylaminoethyl analog possessed local anesthetic activity, though considerably less than cocaine.

The work was extended by Denton and coworkers (8) who prepraed 22 β-aminoketones and evaluated them pharmacologically. In subsequent work by these investigators in which 23 additional β-aminoketones were prepared (9), it was concluded that, in general, a decrease in activity results from: (*a*) increasing the complexity of the amino group, and (*b*) the introduction of more complex aromatic radicals than the phenyl group.

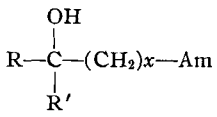
Mannich bases from benzalacetone were prepared by Burckhalter and Johnson (10) as vinylogs of β-aminopropiophenones in an attempt to enhance their analgetic activity. While these compounds were devoid of analgetic activity, they did possess *in vitro* action against bacteria. Denton and co-workers (11) reported the preparation of a secondary alcohol, 3-(1-piperidyl)-1,2-diphenyl-1-propanol hydrochloride, and found that it ranked higher in antispasmodic activity than did the seven tertiary alcohols with which it was compared.

Mannich (12) was not able to obtain *m*-aminophenyl β-piperidinoethyl ketone monohydrochloride by chemical reduction of the nitro base; Wright and Freifelder (13) were able to obtain this compound, however, by catalytic reduction of the ketone monohydrochloride in aqueous solution with 5% palladium on activated charcoal.

Nobles (14) reported the preparation of 3-dimethylamino-1-phenyl-1-propanol hydrochloride by the reduction of the corresponding amino ketone with sodium borohydride.

Received April 28, 1961, from the Graduate School, University of Mississippi, University.
Accepted for publication June 30, 1961.
Abstracted in part from a thesis presented to the Graduate School of the University of Mississippi by Fred C. Rogers, in partial fulfillment of the requirements of the degree of Master of Science, August 1960.
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Denton and co-workers (15–19) have prepared more than 100 γ-amino tertiary alcohols, a majority of which have exhibited physiological activity. The general structure of the γ-amino-alcohols prepared by these workers is represented as follows

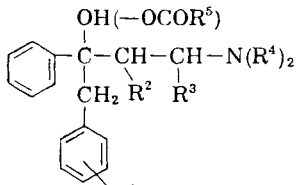


Cunningham and co-workers (20) prepared 3-(N - piperidyl) - 1 phenyl - 1 - cyclohexy - 1 - propanol hydrochloride[1] and related compounds. These compounds were found to be potent antispasmodics.

Adamson (21) found that the intermediates for the preparation of compounds with antihistaminic properties may be obtained by treating tertiary aminoethyl ketones or their salts with 2-pyridyllithium and hydrolyzing the organo-lithium compound formed. One of the compounds mentioned was a 2-thiophene derivative: 3-dimethylamino-1-(2-thienyl)-1-propanol.

Pohland and Sullivan (22) demonstrated the formation of tertiary alcohols from the reaction of a β-aminoketone with a Grignard reagent and hydrolysis of the addition product to form 4-dimethylamino - 1,2 - diphenyl - 3 - methyl - 2-butanol and its hydrochloride salt and related compounds.

Pohland and Sullivan (22) were interested in the preparation of 4-dialkylamino-1,2-diphenyl-2-butanols and their esters. The general structure of these compounds may be represented as



Preliminary pharmacological evaluation of *dl*-4 - dimethylamino - 1,2 - diphenyl - 3 - methyl - 2-propionyl oxybutane hydrochloride indicated that the α-isomer possessed a high order of analgesic activity in animals and was found to be an effective analgesic in human beings (23).

DISCUSSION

The preparation of three β-aminoketones, 3-dimethylamino - 2 - methyl - 1 - (2 - thienyl) - 1 - propanone hydrochloride and the 3-piperidyl and 3-morpholinyl analogs, was accomplished by the reaction of 2-propionylthiophene and paraformaldehyde with the appropriate secondary amine hydrochloride. The starting compound, 2-propionylthiophene, was prepared by following the method of Kosak and Hartough (24) for the preparation of 2-acetylthiophene.

A different procedure was followed in the synthesis of each of the three β-aminoketones rather than following one general procedure. 3-Dimethylamino-2-methyl-1-(2-thienyl)-1-propanone had previously been prepared by Blicke and Burckhalter (25). Harradence and Lions (26) described a procedure for the production of Mannich bases using morpholine hydrochloride. This method was followed in the synthesis of 4-morpholinyl-3-methyl-1-(2-thienyl)-1-propanone hydrochloride. The method of Levvy and Nisbet (7) was utilized for the synthesis of the 4-piperidyl analog.

As a result of the relatively low yield (33%) of 4-piperidyl-3-methyl-1-(2-thienyl)-1-propanone hydrochloride obtained by following the method outlined by Levvy and Nisbet, the present authors found it necessary to prepare an additional quantity of this Mannich base salt. The method of Maxwell (27) was followed for this second preparation and a yield of 49% was obtained. Levvy and Nisbet (7) stated that a 1 molar quantity of paraformaldehyde is used in the initial reaction mixture and the mixture is refluxed for 15 minutes. A second mole of paraformaldehyde is added and refluxing is continued for an additional 15 minutes. Maxwell (27) uses a 1.5 molar equivalent of paraformaldehyde, which is placed in the reaction mixture at the onset of the reaction, and the reaction mixture is refluxed for 3 hours.

Table I indicates pertinent data with respect to the Mannich bases utilized in this study.

The preparation of the three γ-amino secondary alcohols, 3-dimethylamino-2-methyl-1-(2-thienyl)-1-propanol hydrochloride and the 3-piperidyl and 3-morpholinyl analogs, was patterned after the method of Chaikin and Brown (28) as modified by Nobles (14).

The secondary alcohols were obtained by reduction of the corresponding Mannich bases with sodium borohydride. Sodium borohydride is reported (29) to react quite vigorously with aldehydes and ketones at temperatures as low as −40° in methyl alcohol solution, but a quite gentle reaction is obtained at room temperature in an aqueous solution. A moderately vigorous, but easily controllable reaction was obtained at 20–30° when the ketone in methyl alcohol solution was reacted with a 50% methyl alcohol solution of sodium borohydride.

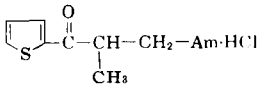
Data with respect to these alcohols are listed in Table II.

The preparation of γ-amino tertiary alcohols involves the reaction of a β-aminoketone with a Grignard reagent. Three different Grignard reagents were utilized in the preparation of this series of compounds: benzylmagnesium chloride, methylmagnesium bromide, and ethylmagnesium bromide. Only the first of the three Grignard reagents was prepared in this laboratory. The remaining two reagents were obtained commercially. The procedure of Gilman and Catlin (30) was followed for the preparation of benzylmagnesium chloride.

The synthesis of 4-dimethylamino-3-methyl-1-phenyl-2-(2-thienyl)-2-butanol hydrochloride and

[1] Marketed as Artane hydrochloride by Lederle Laboratories.

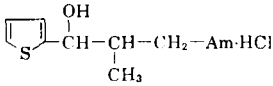
TABLE I.—β-AMINOKETONES



Am	Yield, %	M.p., °C.	Formula	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Dimethylamino	49	155–157[a]	$C_{10}H_{16}ClNOS$			Not analyzed[a]			
Morpholino	67	178.5–180.5	$C_{12}H_{18}SClNO_2$	52.26	52.27	6.58	6.79	5.08	5.15
Piperidino	33[b]	168–170	$C_{13}H_{20}ClNOS$	57.02	56.51	7.36	7.59	5.12	5.49

[a] Blicke and Burckhalter (25) report a melting point of 154–156°. [b] A later synthesis following the method of Maxwell (30) yielded 49%.

TABLE II.—3-DIALKYLAMINO-2-METHYL-1-(2-THIENYL)-1-PROPANOL HYDROCHLORIDE



Am	Yield, %	M.p., °C.	Formula	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Dimethylamino	48[a]	152–156	$C_{10}H_{18}ClNOS$	50.94	51.12	7.69	7.84	5.94	5.79
Piperidinyl	83	199–200	$C_{13}H_{22}ClNOS$	56.60	56.34	8.04	7.92	5.08	5.19
Morpholinyl	87	178–181	$C_{12}H_{20}ClNO_2S$	51.87	51.96	7.26	7.38	5.04	4.87

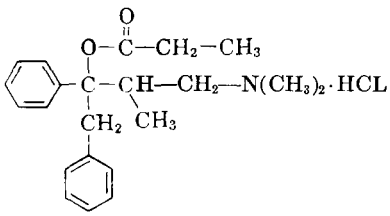
[a] Hydrochloride salt is extremely hygroscopic until washed free of impurities.

the 4-piperidyl and 4-morpholinyl analogs were carried out by following the procedure of Pohland and Sullivan (31). An attempted synthesis of 4-morpholinyl-3-methyl-1,2-diphenyl-butanol hydrochloride was conducted by following the procedure of Denton and his associates (11). The main difference in this method and the method of Pohland and Sullivan is the method of decomposing the addition complex. Denton's method advocates the use of hydrochloric acid to effect the decomposition. The probable reason for the failure of the present authors to obtain the γ-morpholinyl tertiary alcohol is that, after the addition complex was decomposed with dilute hydrochloric acid, the reaction mixture was allowed to stand overnight. The presence of the mineral acid in the reaction mixture for an extended time was undoubtedly sufficient to cause dehydration of the tertiary alcohol. The reaction product gave a positive test for unsaturation when treated with bromide in carbon tetrachloride and aqueous potassium permanganate. This portion of the work will be modified correspondingly in subsequent work.

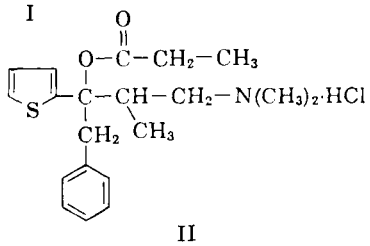
The tertiary alcohols are listed in Table III.

The attempted synthesis of γ-aminoalkyl esters was patterned after the work of Pohland and Sullivan (22, 23, 31). These workers prepared a compound, α - *d* - 4 - dimethylamino - 1,2 - diphenyl - 3 - methyl-2-propionyloxybutane hydrochloride (I).[2] This drug is reported to be an effective analgesic which is comparable to codeine in analgetic activity and is nontoxic and nonaddicting.

Various procedures for the esterification of tertiary alcohols were utilized by the present authors in the attempted synthesis of 4-dimethylamino-3-methyl-1-phenyl-2-propionyloxy-2-(2-thienyl)-butane hydrochloride (II) and the γ-morpholinyl and γ-piperidyl analogs.



I



II

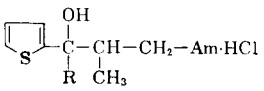
Each attempt met with failure. An examination of the infrared spectrum of each of the reaction products did not reveal the presence of the characteristic alkyl ester absorption in the region of 1735-1750 cm.$^{-1}$ and, in most cases, no absorption peaks of any consequence could be observed from 1650-2000 cm.$^{-1}$.

The reason for the failure to obtain the γ-amino esters is not absolutely clear, although Campaigne and Diedrich (32) stated that their inability to obtain esters of secondary alcohols, derived from 2-acylthiophenes, was due to the relative inactivity of the hydroxyl group. They claimed that this inactivity was due to hindrance of the α-carbon in 2-substituted thiophenes.

Some of the reaction products from the attempted esterifications were tested for unsaturation with bromine in carbon tetrachloride and dilute permanganate solution. The tests indicated that dehydration had occurred. The location of the unsaturation in the olefinic products has not been

[2] Commercially available from Eli Lilly and Co. as Darvon.

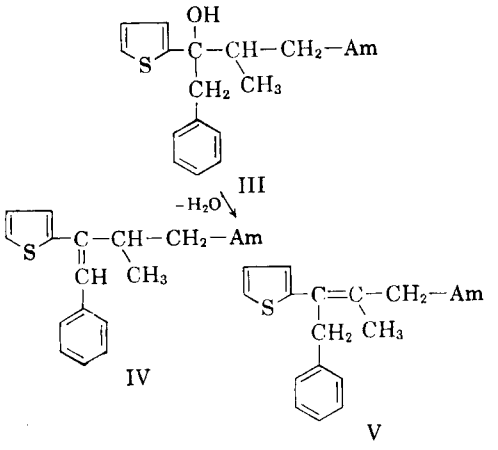
TABLE III.—γ-AMINO TERTIARY ALCOHOLS (HYDROCHLORIDE SALTS)



R	Am	Yield, %	M.p., °C.	Formula	Analyses—Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Methyl	Diethylamino	55	170–172	$C_{11}H_{20}ClNOS$	52.89	52.64	8.07	8.18	5.61	5.52
Methyl	Morpholinyl	68	167–172	$C_{13}H_{22}ClNO_2S$	53.50	53.36	7.60	7.68	4.80	4.91
Ethyl	Dimethylamino	50	174.5–175	$C_{12}H_{22}ClNOS$	54.63	54.58	8.41	8.31	5.31	5.52
Ethyl	Morpholinyl	55	163–167	$C_{14}H_{24}ClNO_2S$	55.12	55.26	7.86	7.76	4.57	4.63
Benzyl	Dimethylamino	57	180–184	$C_{17}H_{24}ClNOS$	62.65	62.80	7.42	7.55	4.29	4.27
Benzyl	Piperidinyl	32	169–177	$C_{19}H_{28}ClNOS$	65.64	65.84	7.71	7.95	3.83	3.59

determined, but, by consideration of resonance stabilization, it would be possible to predict the principle product of the dehydration of III as being that represented by structure IV.

Both the dehydration in preference to esterification and the suggested structure, IV, are in accord with the modern theories of this facet of organic chemistry as suggested by Morrison and Boyd (33). This is true despite the success of Pohland and Sullivan (22) in obtaining the tertiary alcohol esters in the *d*-propoxyphene hydrochloride series.



EXPERIMENTAL

The Mannich bases were prepared according to the general methods already indicated in this paper, according to the well-known methods of the chemical literature.

3 - Dimethylamino - 2 - methyl - 1 - (2 - thienyl)-1 propanol Hydrochloride.—Following the general procedure of Chaikin and Brown (30) as employed by Nobles (14), 10.0 Gm. (0.043 mole) of 3-dimethylamino-2-methyl-1-(2-thienyl)-1-propanone hydrochloride was dissolved in a minimum amount of water and made basic with ice cold 20% sodium hydroxide. The basic mixture was then extracted with ether. The ether was removed by evaporation at the water aspirator, and the free base was dissolved in 50 ml. of methyl alcohol. Meanwhile, 1.615 Gm. (0.427 mole) of sodium borohydride in 100 ml. of 50% methyl alcohol, cooled to 20°, was prepared and placed in a 300-ml. round-bottom flask, equipped with a magnetic stirrer, reflux condenser, and dropping funnel. The flask containing the sodium borohydride solution was surrounded by a cooling bath, and the alcoholic solution of the aminoketone was added, dropwise, with stirring and cooling so as to maintain the temperature below 30°. After the addition was complete, the mixture was stirred for an additional 30 minutes. The flask was removed from the cooling bath and warmed to 40–50° so as to decompose the excess sodium borohydride. The solution was then concentrated *in vacuo* to remove the alcohol. The residue was ether extracted and dried overnight with anhydrous magnesium sulfate. The drying agent was removed by filtration. The ether extract was chilled by means of an ice-water bath and treated with anhydrous hydrogen chloride while being stirred with a magnetic stirrer. The hydrochloride salt was collected on a sintered-glass funnel and washed with ether. Recrystallization from ethyl alcohol-ethyl acetate yielded 4.8 Gm. (48%) of white crystals, m.p. 152–156°.

3 - Piperidyl - 2 - methyl - 1 - (2 - thienyl) - 1-propanol Hydrochloride.—Following the above described procedure, 2.8 Gm. (0.074 mole) of sodium borohydride in 150 ml. of 50% methyl alcohol and 17.3 Gm. (0.073 mole) of 3-piperidyl-2-methyl-1-(2-thienyl)-1-propanone in 100 ml. of methyl alcohol yielded 16.8 Gm. (83%) of white crystals, m.p. 150–185°. A small sample, after recrystallization from ethyl alcohol-ethyl acetate, melted at 199–200°.

3 - Morpholinyl - 2 - methyl - 1 - (2 - thienyl) - 1-propanol Hydrochloride.—By the method outlined previously, 1.37 Gm. (0.036 mole) of sodium borohydride in 75 ml. of 50% methyl alcohol and 8.7 Gm. (0.036 mole) of 3-morpholinyl-2-methyl-1-(2-thienyl)-1-propanone in 75 ml. of methyl alcohol yielded 6.7 Gm. (67%) of white crystals, m.p. 172–180°. A sample after recrystallization from ethyl alcohol-ethyl acetate, melted at 178–181°.

4 - Dimethylamino - 3 - methyl - 2 - (2 - thienyl)-2-butanol Hydrochloride.—The general method of Pohland and Sullivan (23) for the preparation of γ-amino tertiary alcohols was used for this preparation. In a dry 500-ml. three-neck flask fitted with a mercury-sealed stirrer, a dropping funnel, and a condenser provided at its upper end with a drying tube containing a mixture of calcium chloride and soda lime, was placed 0.214 mole of methylmagnesium bromide and 50 ml. of dry ether. 3-Dimethylamino-2-methyl-1-(2-thienyl)-1-propanone, 21.1 Gm. (0.107 mole) in 200 ml. of dry ether was

pipetted into the dropping funnel and added dropwise with stirring to the Grignard solution. After the addition was completed, the reaction mixture was refluxed with stirring for 1 hour and cooled to room temperature. The reaction mixture was decomposed by the dropwise addition of a saturated ammonium chloride solution. The ether solution was decanted from the granular solid and the solid material was washed with 100 ml. of ether. The combined ether solutions were dried overnight with anhydrous magnesium sulfate. The drying agent was removed by filtration, and the ether solution was treated with anhydrous hydrogen chloride. The hydrochloride salt formed as a sticky mass and adhered to the walls of the flask. The ether was quickly decanted from the sticky mass and 10 ml. of acetone was added to the mass. The flask was restoppered and allowed to stand for 1 hour. After the mass had solidified, it was collected on a sintered-glass funnel and washed with a small quantity of acetone yielding 15.3 Gm. (55%) of white crystals m.p. 160–167°. A sample, after recrystallization from ethyl alcohol, melted at 170–172°.

4 - Morpholinyl - 3 - methyl - 2 - (2 - thienyl)-2-butanol Hydrochloride.—Following the above described procedure, 0.15 mole of methylmagnesium bromide in 50 ml. of dry ether and 16.15 Gm. (0.068 mole) of 3-morpholinyl-2-methyl-1-(2-thienyl)-1-propanone in 150 ml. of dry ether yielded 13.4 Gm. (68%) of white crystals, m.p. 160–166°. A sample, after recrystallization from ethyl alcohol, melted at 167–172°.

1-Dimethylamino - 2-methyl-3-(2-thienyl)-3-pentanol Hydrochloride.—By the method outlined previously, 0.214 mole of ethylmagnesium bromide in 50 ml. of dry ether and 21.1 Gm. (0.107 mole) of 3-dimethylamino-2-methyl-1-(2-thienyl)-1-propanone in 200 ml. of dry ether yielded 14.0 Gm. (50%) of white crystals, m.p. 170–173°. A sample, after recrystallization from ethyl alcohol-acetone, melted at 174.5–175°.

1 - Morpholinyl - 2 - methyl - 3 - (2 - thienyl) - 3-pentanol Hydrochloride.—By the method outlined previously, 0.182 mole of ethylmagnesium bromide in 50 ml. of ether and 21.7 Gm. (0.091 mole) of 3-morpholinyl-2-methyl-1-(2-thienyl)-1-propanone in 150 ml. of dry ether yielded 15.3 Gm. (55%) of white crystals, m.p. 154–160°. A sample, after recrystallization from ethyl alcohol-acetone, melted at 163–165°.

4 - Dimethylamino - 3 - methyl - 1 - phenyl - 2-(2-thienyl)-2-butanol Hydrochloride.—By the previously described method of Gilman and Catlin (31), 0.152 mole of benzylmagnesium chloride was prepared in a 2-L. three-neck flask equipped with a mercury-sealed stirrer, a dropping funnel, and a condenser provided with a drying tube containing a mixture of calcium chloride and soda lime. Following the method of Pohland and Sullivan (23) for the preparation of the tertiary alcohol, the Grignard solution was stirred during the dropwise addition of a solution of 14.9 Gm. (0.76 mole) of 3-dimethylamino-2-methyl-1-(2-thienyl)-1-propanone in 200 ml. of dry ether. After the addition of the ketone was completed, the reaction mixture was refluxed for 1 hour and then allowed to cool to room temperature. The reaction mixture was decomposed by the dropwise addition of a saturated ammonium chloride solution. The ether was decanted from the granular solid, and the solid material was washed with 50–75 ml. of ether. The combined ether solutions were dried overnight with anhydrous magnesium sulfate. The drying agent was removed by filtration, and the ether solution was treated with anhydrous hydrogen chloride. The white precipitate was collected on a sintered-glass funnel yielding 14 Gm. (57%) of white crystals, m.p. 156–165°. A sample, after recrystallization from methyl alcohol-ethyl acetate (1:3 ratio) melted at 180–184°.

4 - Piperidyl - 3 methyl - 1 - phenyl - 2 - (2-thienyl)-2-butanol Hydrochloride.—By the method outlined above, 0.38 mole of benzylmagnesium chloride and 30.0 Gm. (0.126 mole) of 3-piperidyl-2-methyl-1-(2-thienyl)-1-propanone in 200 ml. of dry ether yielded 15 Gm. (32%) of white crystals, m.p. 120–170°. A sample, after recrystallization from methyl alcohol-ethyl acetate, melted at 169–177°.

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Some Aspects of Inhaler Technology

By LLOYD KENNON and JOHN J. GULESICH

This communication presents the results of a physical-chemical study of the characteristics of the inhaler dosage form. Such a study has not been reported previously. Analysis of the observations made permit the following generalizations: inhalers may be classified as being either a surface type (provides zero-order release) or a solution type (provides first-order type of release); in inhaler development the rates of volatilization can be controlled or modified; vapor pressure equilibrium concepts play a minor role. These concepts are amplified and discussed with supporting data.

INFORMATION concerning inhaler technology is conspicuous by its absence in readily available sources. The U.S.P. XVI (p. 823), which contains only one inhaler monograph (propylhexedrine inhalant), describes this dosage form as follows: "Inhalants are drugs or combinations of drugs which, by virtue of their high vapor pressure, can be carried by an air current into the nasal passage where they exert their effect. The device making possible the administration of an inhalant is known as an inhaler."

Inhalers, like all pharmaceutical products, should be physically and chemically stable so that satisfactory shelf- and use-life is attained. However, inhalers present somewhat different problems in that the delivered doses are not easily standardized, unlike the cases involving tablets, capsules, or solutions. In fact, the precision with which inhaler doses can be made "uniform" is of a completely different order of magnitude from that attainable with the latter products.

The purpose of this paper is to show that inhaler technology and inhaler problems can be profitably examined by applying the principles of physical chemistry. It is possible by these means to obtain a clearer picture of the behavior, including the limitations, of inhaler dosage forms. Variables such as the shape and size of the inhaler body and pledget, the concentration and state of the pledget charge, and the variation in performance of both individual and groups of inhalers have been studied. No attempt will be made here to discuss all the medical and pharmaceutical aspects or the chemical reactivities which would have to be considered in the formulation of a complete inhaler. Such factors as the physical and chemical stability, solubility, mechanical integrity, or compatibility of the active ingredient with the inhaler body, pledget, solvent, or organoleptic agents will not be discussed.

The paper describes and discusses some of the data obtained during the development of an inhaler whose volatile ingredient was phlorone, 2,5-dimethyl-1,4-benzoquinone. The inhaler was to deliver at least 50 mcg. of phlorone per liter of air passing through it.

EXPERIMENTAL

Apparatus.—Figure 1 pictures the breathing apparatus used in the evaluation of the inhalers. In essence, the system is designed to pull a known amount of air through an inhaler and then through a quantity of liquid in which the active ingredient in the vapor dissolves and which can be analyzed. The device was set so that each cycle took 2 seconds and delivered 200 ml. of air. Because the size of the holes in the glass aerating head affected the quantity of air which would pass per cycle, the apparatus was calibrated with a Collins vitalometer.[1] This was done by inflating the vitalometer, then connecting it in the position at which the inhaler is put during testing, running the breathing apparatus, and noting the decrease in air volume of the vitalometer per breathing cycle.

Procedure.—The manipulations consisted of drawing air serially through the various inhaler formulations and then through 25 ml. of analytical reagent grade isopropanol contained in a 75-ml. test tube. The isopropanol solution was then analyzed on the Beckman DU spectrophotometer. A second tube of isopropanol in series showed that no phlorone was carried out as vapor from the first tube, so only one tube was needed. The pump or breathing apparatus was set so that 200 ml. of air was pumped per complete cycle, each cycle taking 2 seconds (the actual time of air flow then being 1 second, with the machine "exhaling" the other second). To put 1 L. of air through an inhaler then took, of course, five cycles or 10 seconds.

Analytical.—Many standard solutions of phlorone in analytical reagent grade isopropanol were made and run at 250 mμ on the Beckman DU instrument; the response was linear and reproducible to a very

Received October 11, 1961, from Smith Kline and French Laboratories, Pharmaceutical Chemistry Section, Philadelphia 1, Pa.
Accepted for publication January 3, 1962.

[1] A. H. Thomas Co., Phila. 5, Pa.; Scientific Apparatus and Reagents Catalog, No. 6433, 1961 Anniversary Edition.

TABLE I.—DOSE OBTAINED FROM SURFACE PHLORONE INHALERS UTILIZING BLOTTER AND COTTON PLEDGETS

Inhaler No.	Pledget[a]	Air, L.	Dose, mcg.	Inhaler No.	Pledget[a]	Air, L.	Dose, mcg.
1	B	1	104	12	B	2	138
2	B	1	106	13	B	1	87
3	B	1	108	14	B	1	73
4	B	1	119	15	B	3	182
5	B	1	117	16	B	3	175
6	B	1	108	17	C	2	95
7	B	1	104	18	C	2	73
8	B	1	110	19	C	1	58
9	B	1	115	20	C	1	58
10	B	1	135	21	C	3	80
11	B	2	152	22	C	3	118

[a] B, paper blotter, folded, 100-lb. white, J. L. N. Smythe Co., Philadelphia, Pa.; C, cotton pledget.

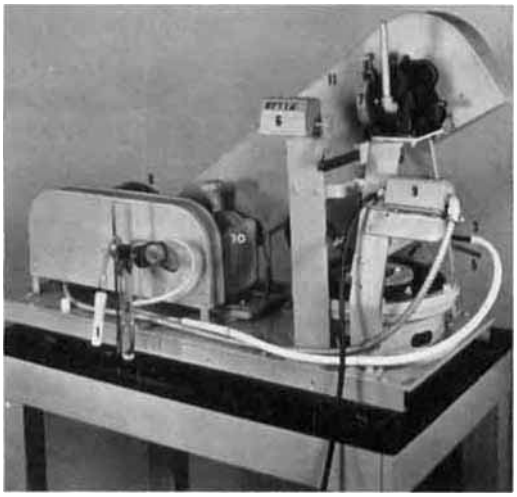


Fig. 1.—Breathing apparatus; *1*, inhaler; *2*, tube, contains aerating head and isopropanol; *3*, air inlet; 4, rubber bellows-diaphragm; *5*, air outlet; *6*, counter; *7*, cam, attached by descending rod to bellows-diaphragm to permit volume adjustment; *8*, motor; *9*, motor switch; *10*, V-belt drive with variable sheave to permit speed adjustment; *11*, belt drive, rotates cam wheel through right-angle transmission.

high degree of precision. Solutions stored in the dark for various periods of time up to 8 days assayed the same as originally. The absorptivity or absorbancy index was found to be 139; $K = 139 =$ absorbance/(Gm./L.). Since 25 ml. was a convenient volume of isopropanol in which to collect phlorone vapors, the relationship became simply: micrograms/25 ml. = absorbance × 180. This assay procedure was found to be very satisfactory. When equal concentrations of camphor, menthol, or eucalyptol were added to the phlorone standard solutions, no interference was noted in the absorbance at 250 mμ.

Materials.—Phlorone or 2,5-dimethyl-1,4-benzoquinone, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y. Benzyl salicylate, Givaudan-Delawanna, Inc., New York, N. Y. Dibutyl phthalate, Matheson, Coleman and Bell, Division of the Matheson Co., Inc., East Rutherford, N. J. Paper-blotter, 100-pound, white, J. L. N. Smythe Co., Philadelphia, Pa. Long and short size inhaler bodies, nose-pieces, caps, and cotton pledgets as shown in Fig. 2. Isopropyl alcohol, analytical reagent, Mallinckrodt Chemical Works, St. Louis, Mo. Eucalyptol U.S.P., Dodge and Olcott, Inc., New York, N. Y. Camphor U.S.P., S. B. Penick and Co., New York, N. Y. Menthol U.S.P., racemic, Givaudan-Delawanna, Inc., New York, N. Y.

RESULTS AND DISCUSSION

Initial Studies.—The first inhalers were made by dissolving the phlorone in ether, placing the appropriate number of pledgets in the solution, and then evaporating the ether. The ether evaporating process is unwieldy and results in uneven distribution of the phlorone on the surface of either the cotton or folded blotter pledgets. Table I presents some of the data taken on inhalers which contained 50 mg. of phlorone and 4 mg. of menthol made by this process. The long plastic body was used. The greater available surface area of the blotter provided somewhat higher doses than the cotton pledget.

To replace the ether evaporation procedure, phlorone was added to the pledgets in solution form. Two solvents of very low volatility were chosen for study: dibutyl phthalate and benzyl salicylate. A solubility study was done to determine the limits of concentration that could be used. The limits determined were: 50 mg./ml. in dibutyl phthalate and 85 mg./ml. in benzyl salicylate. The solutions made with the latter formed more easily.

A group of inhalers was made by putting 1 ml. of a 50 mg./ml. solution of phlorone in dibutyl phthalate on cotton and blotter pledgets encased in the long plastic body. Doses obtained from this group are given in Table II. Again it is seen that the greater exposed surface area of the blotter pledget provides the higher doses.

TABLE II.—DOSE OBTAINED FROM SOLUTION PHLORONE INHALERS UTILIZING BLOTTER AND COTTON PLEDGETS

Inhaler No.	Pledget	Dose, mcg./2 L. Air	Inhaler No.	Pledget	Dose, mcg./2 L. Air
1	Cotton	98	7	Blotter	150
2	Cotton	77	8	Blotter	135
3	Cotton	76	9	Blotter	135
4	Cotton	80	10	Blotter	135
5	Cotton	80	11	Blotter	119
6	Cotton	80	12	Blotter	133

TABLE III.—COMPARISON OF DOSES OBTAINED FROM NINE DIFFERENT GROUPS OF INHALERS

Inhaler No.	Inhaler Group	Dose, mcg./L. of Air	Inhaler No.	Inhaler Group	Dose, mcg./L. of Air	Inhaler No.	Inhaler Group	Dose, mcg./L. of Air
1	A	76	13	D	89	25	G	110
2	A	78	14	D	90	26	G	66
3	A	76	15	D	88	27	G	71
4	A	82	16	D	93	28	G	67
5	B	83	17	E	57	29	H	57
6	B	65	18	E	62	30	H	44
7	B	81	19	E	72	31	H	41
8	B	102	20	E	62	32	H	50
9	C	93	21	F	92	33	I	94
10	C	101	22	F	90	34	I	81
11	C	131	23	F	91	35	I	84
12	C	91	24	F	101	36	I	87

TABLE IV.—AVERAGE DOSES FROM INHALER GROUPS OF TABLE III

Inhaler Group	Dose, mcg./L. of Air	Inhaler Group	Dose, mcg./L. of Air	Inhaler Group	Dose, mcg./L. of Air
A	78	B	83	C	95[a]
D	90	E	63	F	93
G	68[b]	H	48	I	86

[a] Neglecting reading of 131 as being somewhat out of line for purposes of obtaining these initial impressions. [b] Neglecting reading of 110, same reason.

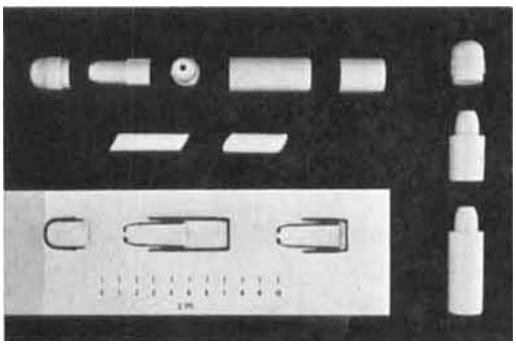


Fig. 2.—External and cut-away views of inhaler components.

On the basis of these data (Tables I and II) more inhaler groups were made in an attempt to decide which type of pledget and which inhaler type (surface or solution) would give better results. The following nine groups, all in long bodies, were then made: Surface: (*A*) evaporated ether method, 50 mg. charge, blotter pledget; (*B*) same method, long cotton pledget; (*C*) same method, short cotton pledget. Solution: (*D*) method: 1 ml. of warm 100 mg./ml. solution of phlorone in benzyl salicylate per pledget, blotter pledget; (*E*) method same, long cotton pledget; (*F*) method same, short cotton pledget; (*G*) method: 1 ml. of 50 mg./ml. solution of phlorone in dibutyl phthalate per pledget, blotter pledget; (*H*) same method, long cotton pledget; (*I*) same method, short cotton pledget.

The data obtained from these nine groups are given in Tables III and IV.

Taking an overview of the data of Table III, we may look at the averages as presented in Table IV.

Besides these data, trial runs with other Group A and B inhalers were made. Fifteen analyses (three inhalers breathed five times each at half-hour intervals) from each group showed that Group A gave an average dose of 78 mcg./L. of air; Group B gave 72 mcg./L. of air.

The data indicate that, all things considered (dose level, ease of making, solubility, etc.), the short cotton pledget charged with phlorone in benzyl salicylate appears to produce the best inhaler. We recognize that the "package" is an inherent part of the formula in an inhaler dosage form because the internal size and shape may govern the amount of drug put into the formula and the dosage obtained per "whiff."

Although inhalers made by the "evaporated ether" process were of less interest, some limited usage tests were made using inhalers of this type. The same four inhalers of Group A used to obtain the data of Table III were also used here. The time of breathing is also shown along with the dose data in Table V.

The data of Table V provide an insight into how well and with what variation some of the original inhalers perform.

Because of the possibility that more efficient utilization of the drug might result from its application closer to the nasal openings so that more of the inner nasal surfaces would be bathed with the vapors, some larger, aluminum inhaler bodies (identical to those of the obsolete Benzedrine inhaler) were tested. These bodies are somewhat thicker, and the nose-piece is of such size that it does not easily penetrate the nose as far as the nozzles shown in Fig. 2. It was found that the airflow characteristics of this inhaler body are such that the pledgets supply a dose lower than they do when encased in the plastic bodies. Even when four extra holes were drilled (No. 50 drill) in the sides of the air-inlet end in an attempt to permit more air to pass through, no increase in dosage was obtained. This indicates that resistance to air flow can be a rate-limiting factor. Table VI presents the data obtained.

Final Studies.—On the basis of the data collected on the preliminary inhalers, further work was done only on solution-type inhalers. Tables VII–IX present some of the data taken on inhalers containing 85 mg. of phlorone and 10, 20, or 30 mg. of camphor dissolved in 1 ml. of benzyl salicylate. The short size body and pledget was used. The camphor odor persisted throughout the trials. The data indicated also that the phlorone dosage is not affected by the presence of various amounts of camphor.

TABLE V.—LIMITED USAGE TEST OF SURFACE INHALERS OF TABLE III, GROUP A

Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air
1	1	..	45	1	2	1	123	1	3	1	61
2	1	..	54	2	2	1	73	2	3	1	62
3	1	..	72	3	2	1	73	3	3	1	66
4	1	..	71	4	2	1	45	4	3	1	71
1	1	1	95	1	2	1	63	1	3	1	60
2	1	1	101	2	2	1	58	2	3	1	58
3	1	1	89	3	2	1	68	3	3	1	68
4	1	1	72	4	2	1	65	4	3	1	71
1	1	1	138	1	2	1	99	1	3	2	57
2	1	1	84	2	2	1	65	2	3	2	56
3	1	1	85	3	2	1	69	3	3	2	69
4	1	1	78	4	2	1	65	4	3	2	72
1	1	1	105	1	2	1	53	1	3	1.5	56
2	1	1	70	2	2	1	53	2	3	1.5	57
3	1	1	86	3	2	1	53	3	3	1.5	67
4	1	1	75	4	2	1	58	4	3	1.5	68
1	1	1	67	1	2	1	56	1	3	1	54
2	1	1	66	2	2	1	59	2	3	1	55
3	1	1	79	3	2	1	62	3	3	1	70
4	1	1	58	4	2	1	61	4	3	1	66
1	1	1	129	1	2	1	112	1	4	..	58
2	1	1	87	2	2	1	60	2	4	..	64
3	1	1	91	3	2	1	55	3	4	..	77
4	1	1	78	4	2	1	58	4	4	..	65
1	1	1	114	1	2	1	52	1	4	1	56
2	1	1	79	2	2	1	52	2	4	1	58
3	1	1	75	3	2	1	52	3	4	1	67
4	1	1	65	4	2	1	62	4	4	1	68
1	1	1	120	1	3	..	62	1	4	3	57
2	1	1	66	2	3	..	62	2	4	3	61
3	1	1	78	3	3	..	53	3	4	3	..
4	1	1	72	4	3	..	60	4	4	3	72
1	2	..	86	1	3	1	58	1	4	2	58
2	2	..	59	2	3	1	65	2	4	2	58
3	2	..	63	3	3	1	69	3	4	2	68
4	2	..	65	4	3	1	68	4	4	2	78

Although the data are given in quite a detailed manner, attempts to summarize the information result in too great a loss of clarity and completeness in the total picture.

Because rate processes should control inhaler function, it is interesting to note the effect of temperature on the rate of volatilization of phlorone. Several inhalers from the groups used to obtain the data of Tables VIII and IX were heated to 37°, then breathed. It can be seen that this increase in temperature increases the dose about 80% over that obtained at room temperature. Table X presents the data.

The data shown in Table XI resulted from study of the following solution-type inhaler formula: phlorone, 85.0 mg.; eucalyptol U.S.P., 0.03 ml.; and benzyl salicylate *q.s. ad.* 1.0 ml.

The phlorone and eucalyptol are dissolved in the benzyl salicylate, then 1 ml. of the solution is dropped onto the small cotton pledget which is then encased in the body of the inhaler. Some less extensive data on similar formulas (same except having 0.01 or 0.02 ml. of eucalpytol) were also collected but are not given here because they essentially duplicate the data following. The eucalyptol odor persisted throughout the trials.

The data of Table XI corroborate the concept that rate processes exert more weight than equilibrium does. For example, one of the continuous breathing series, dose numbers 122–150, shows that recovery times are essentially nonexistent.

In addition to the data already presented, this table also permits one (by examining the vertical columns) to see the variation in the performance of individual inhalers. Even though the ranges are fairly wide, the inhalers do perform quite consistently on the average. The following are the average doses given by the Table XI inhalers: No. 1, 85; No. 2, 90; No. 3, 83; No. 4, 91; No. 5, 84.

To check the effect of the state of the total charge the following was done: two groups of inhalers were made, each inhaler containing 42.5 mg. of phlorone and 0.015 ml. of eucalyptol. However, one group had the ingredients dissolved in 0.5 ml. of benzyl salicylate, the other in 1.0 ml. Each of the ten inhalers (short body, cotton pledget) was breathed six times with the following results: the "0.5-ml. group" averaged 64 mcg./L. of air, whereas the "1.0 ml. group" averaged 37. The conclusion is that in a solution type inhaler the concentration of drug is of much greater importance than the surface area over which it is spread. Again, this concentration dependence indicates that rate rather than equilibrium factors predominate.

We further investigated the effect of charge concentration on delivered dose. Phlorone-eucalyptol-benzyl salicylate inhalers were made having phlorone concentrations of 80, 70, 60, 50, and 40 mg./ml. in benzyl salicylate. Each short cotton inhaler pledget was charged with 1 ml. of solution. Figure 3 illustrates the results. It can be seen that

TABLE VI.—DOSE OBTAINED FROM SHORT COTTON PLEDGETS ENCASED IN THE BENZEDRINE INHALER ALUMINUM BODY, SOLUTION TYPE INHALER, BENZYL SALICYLATE SOLVENT[a]

Inhaler No.	Dose, mcg./L. of Air	Inhaler No.	Dose, mcg./L. of Air	Inhaler No.	Dose, mcg./L. of Air
1	61	7	56	13	54
2	60	8	52	14	64
3	57	9	61	11	49
4	53	10	55	12	53
5	56	7	75	13	55
6	50	8	56	14	55
7	64	9	64	11	49
8	53	10	52	12	54
9	62	11	58	13	62
10	54	12	69	14	69
7	46	13	67	11	48
8	53	14	68	12	52
9	60	11	56	13	56
10	52	12	55	14	65
7	50	13	63	11	46
8	61	14	53	12	48
9	58	11	52	13	51
10	54	12	57	14	60
7	51	13	60	15	53
8	52	14	64	16	51
9	56	11	51	17	55
10	53	12	52	18	55
7	44	13	52	15	46
8	44	14	57	16	45
9	62	11	57	17	51
10	50	12	58	18	54
7	50	13	49	15	46
8	50	14	65	16	48
9	64	11	68	17	51
10	59	12	63	18	56

[a] Inhalers had doses taken from them at half-hour intervals; inhalers No. 15–18 were modified by drilling four holes (No. 50 drill) into side of inlet end.

the inhaler life and dosage level are predictable when such data are available.

The following was done to check the behavior of a surface type inhaler. Inhalers (blotter pledget, long body) were made by the evaporated ether method containing 10, 20, 30, 40, 50, 60, and 70 mg. of phlorone. Figure 4 illustrates the results. It can be seen that in this case the dosage does not fall off as quickly as with the solution type inhaler.

As a comparison to the above and to fortify still further the conclusions drawn from the data of Fig. 3, more inhalers were made containing 40, 50, 60, 70, and 80 mg. of phlorone dissolved in 1 ml. of benzyl salicylate. This time blotter pledgets in the large inhaler body were used. Figure 5 presents the data obtained. Again, just as in Fig. 3, the line goes through the origin.

The data of Figs. 3–5, beside contributing to the general picture, enable one to make a calculation of the useful life of the inhaler. It can be seen that the formula would deliver more than 400 doses of at least 50 mcg./L.

CONCLUSIONS AND COMMENTS

In general, it appears that inhalers may be classified as being one of two types. One kind may be called a "surface" type, i.e., the volatile material, *per se*, resides on the surfaces of the

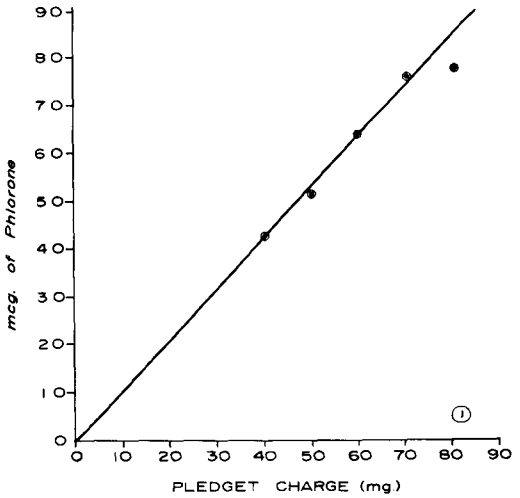


Fig. 3.—Effect of concentration of charge on pledget on dosage delivered when 1 L. of air travels through inhaler (solution type—short cotton pledget).

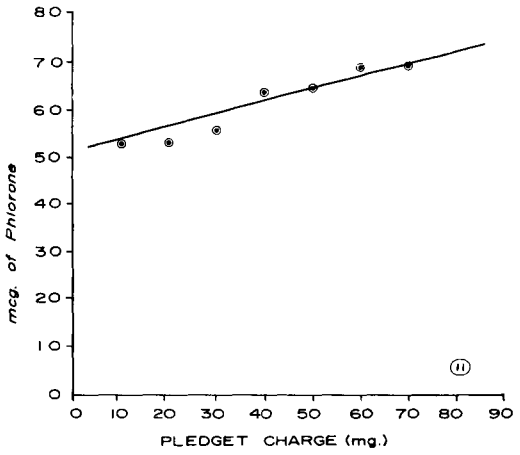


Fig. 4.—Effect of amount of charge on pledget on dosage delivered when 1 L. of air passes through inhaler (surface type—long blotter pledget).

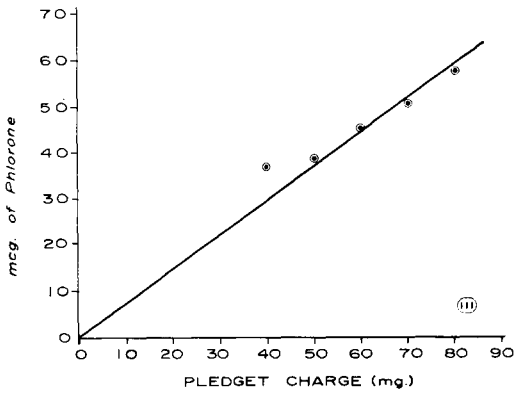


Fig. 5.—Effect of charge concentration on dosage delivered when 1 L. of air passes through inhaler (solution type—long blotter pledget).

TABLE VII.—DOSE OBTAINED FROM PHLORONE (85 MG.)–CAMPHOR (10 MG.)–BENZYL SALICYLATE INHALERS

Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air
1	1	...	87	1	2	0.75	X[a]	1	5	1	X
2	1	...	86	2	2	0.75	84	2	5	1	X
3	1	...	90	3	2	0.75	X	3	5	1	98
4	1	...	90	4	2	0.75	84	4	5	1	X
5	1	...	83	5	2	0.75	X	5	5	1	X
1	1	1	86	1	2	0.75	84	1	6	...	82
2	1	1	85	2	2	0.75	X	2	6	...	X
3	1	1	81	3	2	0.75	80	3	6	...	86
4	1	1	87	4	2	0.75	X	4	6	...	98
5	1	1	87	5	2	0.75	88	5	6	...	93
1	1	3	84	1	2	0.75	X	1	9	...	X
2	1	3	81	2	2	0.75	75	2	9	...	86
3	1	3	75	3	2	0.75	X	3	9	...	83
4	1	3	89	4	2	0.75	83	4	9	...	94
5	1	3	88	5	2	0.75	X	5	9	...	97
1	1	1	83	1	5	...	86	1	9	0.75	X
2	1	1	76	2	5	...	X	2	9	0.75	90
3	1	1	78	3	5	...	X	3	9	0.75	X
4	1	1	82	4	5	...	88	4	9	0.75	93
5	1	1	90	5	5	...	X	5	9	0.75	X
1	2	...	75	1	5	1	X	1	9	1.5	X
2	2	...	80	2	5	1	81	2	9	1.5	X
3	2	...	76	3	5	1	X	3	9	1.5	86
4	2	...	83	4	5	1	X	4	9	1.5	X
5	2	...	86	5	5	1	95	5	9	1.5	85
1	2	1	86	1	5	1	X	1	12	...	X
2	2	1	X	2	5	1	X	2	12	...	91
3	2	1	81	3	5	1	82	3	12	...	X
4	2	1	X	4	5	1	X	4	12	...	96
5	2	1	95	5	5	1	X	5	12	...	X
1	2	1	X	1	5	1	94	1	12	7.5	X
2	2	1	81	2	5	1	X	2	12	7.5	X
3	2	1	X	3	5	1	X	3	12	7.5	87
4	2	1	87	4	5	1	87	4	12	7.5	X
5	2	1	X	5	5	1	X	5	12	7.5	108
1	2	2.75	82	1	5	1	X	1	12	0.5	X
2	2	2.75	X	2	5	1	114	2	12	0.5	100
3	2	2.75	78	3	5	1	X	3	12	0.5	X
4	2	2.75	X	4	5	1	X	4	12	0.5	101
5	2	2.75	91	5	5	1	103	5	12	0.5	X

[a] X indicates that the inhaler was breathed but not assayed.

pledget. This represents a conventional adsorption situation; it is easy to appreciate the fact that the more surface area the pledget has the greater the surface area of the material exposed to the airflow and the greater the opportunity for volatilization. Thus a larger or more loosely packed pledget will cause a larger dose to emanate from an inhaler than a smaller or tightly packed pledget. It is convenient to make this type of inhaler if the volatile material itself is a liquid. It should also be pointed out that the doses produced from a surface type inhaler stay relatively high while the pledget charge is being depleted according to a zero-order scheme. This is reasonable when one visualizes that the volatile material has formed a multimolecular (as distinguished from a monomolecular) layer on the pledget surfaces; thus, even though molecules are stripped off, the surface area and hence the dose remain essentially unchanged. However, as some areas of the pledget are denuded, the total exposed surface area of the volatile material decreases, and so does the dose.

The second inhaler type may be termed a "solution" type, i.e., the volatile material is dissolved in a suitable nonvolatile solvent, and this solution is placed on the pledget. The situation may be taken as an example of the operation of Raoult's and Henry's laws, i.e., the vapor pressure of the components are proportional in some way to their concentrations expressed as mole fractions. To keep the vapor pressure contribution of the solvent low in order to enhance the vapor pressure of the solute, a solvent of very low vapor pressure is used as the vehicle. In this inhaler type the exposed surface area of the material does not change as the inhaler is used; what does change is the concentration of the volatile material in the solvent. Thus the dose gradually decreases according to a first-order scheme as the drug concentration decreases. Of course, the nature of the pledget and the inhaler body

TABLE VIII.—DOSE OBTAINED FROM PHLORONE (85 MG.)–CAMPHOR (20 MG.)–BENZYL SALICYLATE INHALERS

Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air
1	1	...	97	1	2	0.75	95	1	5	1	102
2	1	...	95	2	2	0.75	X[a]	2	5	1	X
3	1	...	91	3	2	0.75	91	3	5	1	X
4	1	...	90	4	2	0.75	X	4	5	1	98
5	1	...	85	5	2	0.75	96	5	5	1	X
1	1	1	93	1	2	0.75	X	1	6	...	98
2	1	1	86	2	2	0.75	80	2	6	...	100
3	1	1	82	3	2	0.75	X	3	6	...	93
4	1	1	91	4	2	0.75	82	4	6	...	95
5	1	1	88	5	2	0.75	X	5	6	...	90
1	1	3	87	1	2	0.75	87	1	9	...	99
2	1	3	85	2	2	0.75	X	2	9	...	94
3	1	3	83	3	2	0.75	85	3	9	...	89
4	1	3	87	4	2	0.75	X	4	9	...	95
5	1	3	89	5	2	0.75	86	5	9	...	91
1	1	1	93	1	5	...	X	1	9	0.75	103
2	1	1	83	2	5	...	86	2	9	0.75	X
3	1	1	91	3	5	...	X	3	9	0.75	94
4	1	1	84	4	5	...	X	4	9	0.75	X
5	1	1	83	5	5	...	87	5	9	0.75	99
1	2	...	80	1	5	1	X	1	9	1.5	X
2	2	...	80	2	5	1	X	2	9	1.5	85
3	2	...	74	3	5	1	100	3	9	1.5	X
4	2	...	81	4	5	1	X	4	9	1.5	95
5	2	...	83	5	5	1	X	5	9	1.5	X
1	2	1	X	1	5	1	91	1	12	...	100
2	2	1	91	2	5	1	X	2	12	...	X
3	2	1	X	3	5	1	X	3	12	...	99
4	2	1	90	4	5	1	93	4	12	...	X
5	2	1	X	5	5	1	X	5	12	...	94
1	2	1	99	1	5	1	X	1	12	7.5	X
2	2	1	X	2	5	1	86	2	12	7.5	93
3	2	1	86	3	5	1	X	3	12	7.5	X
4	2	1	X	4	5	1	X	4	12	7.5	95
5	2	1	95	5	5	1	89	5	12	7.5	X
1	2	2.75	X	1	5	1	X	1	12	0.5	104
2	2	2.75	88	2	5	1	X	2	12	0.5	X
3	2	2.75	X	3	5	1	100	3	12	0.5	95
4	2	2.75	89	4	5	1	X	4	12	0.5	X
5	2	2.75	X	5	5	1	X	5	12	0.5	100

[a] X indicates that the inhaler was breathed but not assayed.

exert some effect here also, because if the airflow through the inhaler and the pledget type do not permit volatilization of the material, insignificant, low doses will result. The phlorone inhaler was of the solution type because the volatile drug is a solid, and solids do not lend themselves to easy pledget charging procedures. The inhaler was designed to deliver in excess of 50 mcg./L. of air passing through. The original doses (when the charge is a full 85 mg.) were about 90 mcg./L. of air; after the equivalent of over 400 doses are delivered and the amount of phlorone drops to 50 mg., the doses were about 52 mcg./L. of air.

If an inhaler were part solution and part surface in nature, one would expect the dosage drop-off line to be steeper than the type exemplified in Fig. 4, yet not as steep as those of Figs. 3 or 5.

Further amplification and clarification of the "surface" and "solution" type classification concept of inhalers might be achieved by considering the existing analogy to chromatographic systems. The surface-type inhaler corresponds to adsorption chromatography wherein the material is initially adsorbed on a carrier, then desorbed by a passing stream of liquid or gas, as the case may be. The solution-type inhaler corresponds to partition chromatography in which material in a solvent is supported by some medium and then partitioned between its original solvent and a passing stream of gas or liquid and thus removed.

Another conclusion which is implicit in the data but somewhat submerged concerns the relationship of the volatile active ingredient to the solvent. An increase in dose should result when the active ingredient is dissolved in solvents which cause it to deviate more positively from Raoult's law. Thus, the less the solute-solvent interaction and the greater the solute-solute interaction, the more pronounced will be the tendency toward volatilization of the solute. Using

TABLE IX.—DOSE OBTAINED FROM PHLORONE (85 MG.)–CAMPHOR (30 MG.)–BENZYL SALICYLATE INHALERS

Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air	Inhaler No.	Time of Day	Breathing Interval, hr.	Dose, mcg./L. of Air
1	1	...	93	1	2	0.75	X[a]	1	5	1	X
2	1	...	90	2	2	0.75	89	2	5	1	105
3	1	...	88	3	2	0.75	X	3	5	1	X
4	1	...	83	4	2	0.75	88	4	5	1	X
5	1	...	79	5	2	0.75	X	5	5	1	107
1	1	1	95	1	2	0.75	86	1	6	...	95
2	1	1	88	2	2	0.75	X	2	6	...	91
3	1	1	87	3	2	0.75	85	3	6	...	92
4	1	1	83	4	2	0.75	X	4	6	...	89
5	1	1	77	5	2	0.75	85	5	6	...	94
1	1	3	89	1	2	0.75	X	1	9	...	90
2	1	3	90	2	2	0.75	86	2	9	...	101
3	1	3	86	3	2	0.75	X	3	9	...	89
4	1	3	84	4	2	0.75	83	4	9	...	95
5	1	3	80	5	2	0.75	X	5	9	...	98
1	1	1	88	1	5	...	X	1	9	0.75	X
2	1	1	90	2	5	...	X	2	9	0.75	102
3	1	1	90	3	5	...	90	3	9	0.75	X
4	1	1	84	4	5	...	X	4	9	0.75	101
5	1	1	79	5	5	...	X	5	9	0.75	X
1	2	...	86	1	5	1	91	1	9	1.5	93
2	2	...	80	2	5	1	X	2	9	1.5	X
3	2	...	82	3	5	1	X	3	9	1.5	95
4	2	...	80	4	5	1	95	4	9	1.5	X
5	2	...	81	5	5	1	X	5	9	1.5	91
1	2	1	94	1	5	1	X	1	12	...	X
2	2	1	X	2	5	1	93	2	12	...	98
3	2	1	91	3	5	1	X	3	12	...	X
4	2	1	X	4	5	1	X	4	12	...	94
5	2	1	93	5	5	1	94	5	12	...	X
1	2	1	X	1	5	1	X	1	12	7.5	96
2	2	1	84	2	5	1	X	2	12	7.5	X
3	2	1	X	3	5	1	91	3	12	7.5	104
4	2	1	91	4	5	1	X	4	12	7.5	X
5	2	1	X	5	5	1	X	5	12	7.5	100
1	2	2.75	92	1	5	1	105	1	12	0.5	X
2	2	2.75	X	2	5	1	X	2	12	0.5	98
3	2	2.75	92	3	5	1	X	3	12	0.5	X
4	2	2.75	X	4	5	1	96	4	12	0.5	94
5	2	2.75	92	5	5	1	X	5	12	0.5	X

[a] X indicates that the inhaler was breathed but not assayed.

TABLE X.—EFFECT OF TEMPERATURE ON LEVEL OF PHLORONE DOSAGE OF INHALERS FROM TABLES VIII AND IX[a]

Inhaler No.	Dose, mcg./L. of Air 25°C.	37°C.	Inhaler No.	Dose, mcg./L. of Air 25°C.	37°C.
VIII-1	95	165	IX-2	92	170
VIII-2	88	194	IX-3	90	159
VIII-3	90	170	IX-4	89	145
VIII-4	90	175	IX-5	89	165
VIII-5	90	152	IX-6	..	150
VIII-6	..	154	IX-7	..	153
VIII-1	95	147	IX-1	92	163
VIII-2	88	152	IX-2	92	165
VIII-3	90	141	IX-3	90	164
VIII-4	90	131	IX-4	89	170
VIII-5	90	138	IX-5	89	162
VIII-6	..	132	IX-6	..	156
IX-1	92	175	IX-7	..	143

[a] Breathing temperature = 25 and 37°. Data are averages of the appropriate figures from Tables VIII and IX.

relative solubility as a gauge of such interaction, one would expect greater doses of phlorone from dibutyl phthalate (in which it is less soluble) than from benzyl salicylate, at the same concentrations. This appears to be the case as comparable inhalers containing phlorone in dibutyl phthalate (50 mg./ml.) give doses of 86 mcg./L. (Table IV, group I), and in benzyl salicylate, 52 mcg./L. (Fig. 3).

Some comments on vapor pressure may be appropriate here. Although it might seem that the vapor pressure of the drug and additives would assume a position of primary importance, this does not appear to be the case. Vapor pressure values represent an equilibrium situation, whereas what is involved in the inhaler cases is a process controlled by factors affecting rates of volatilization. Now it is true of course that volatile materials usually have appreciable vapor pressures. However, it is not generally true that a compound with a vapor pressure value of, e.g., twice that of another compound will volatilize just twice as fast. Besides this fact, inhaler recovery times may be essentially zero, no equilibration time may be needed. Also, no dosage drops would be noted with the surface

TABLE XI.—DOSE OBTAINED FROM PHLORONE INHALERS

Dose No.	Time of Breathing Interval, Day	min.	Dose, mcg./L. of Air Inhaler No. 1	2	3	4	5	Dose No.	Time of Breathing Interval, Day	min.	Dose, mcg./L. of Air Inhaler No. 1	3	5
1	1	...	101	101	101	116	98	60	39	...	78	77	87
2	2	...	94	96	98	93	100	61	39	2	84	97	89
3	2	30	110	109	110	110	102	62	39	1	90	96	88
4	3	...	96	95	X[a]	X	104	63–76	39	2	X	X	X
5	3	40	X	X	102	90	X	77	39	1	91	79	81
6	3	45	103	X	96	X	103	78–97	39	2	X	X	X
7	3	120	X	105	X	98	X	98	39	18	63	63	69
8	4	...	99	102	100	100	95	99–116	39	2	X	X	X
9	4	40	X	X	99	X	X	117	39	1	72	69	69
10	4	30	101	X	X	97	X	118	39	5	75	61	70
11	4	30	X	103	X	X	101	119	39	5	77	65	67
12	24	...	84	79	87	93	90	120	39	98	60	53	57
13	24	5	88	84	90	95	92	121	39	4	57	59	64
14	25	...	75	75	85	85	89	122	51	...	X	X	X
15–23	25	3	X	X	X	X	X	123	51	2	87	98	89
24	25	13	82	81	81	75	81	124	51	2	89	95	91
25–33	25	3	X	X	X	X	X	125–131	51	1.5	X	X	X
34	25	3	75	77	74	70	69	132	51	2	94	81	82
35	28	...	73	76	77	89	77	133	51	2	82	87	87
36–38	28	3	X	X	X	X	X	134–140	51	1	X	X	X
39	28	6	94	98	97	96	91	141	51	29	98	77	74
40–51	28	3	X	X	X	X	X	142	51	2	81	79	84
52	28	24	79	80	79	80	81	143–148	51	1	X	X	X
53–58	28	3	X	X	X	X	X	149	51	27	96	65	64
59	28	1	87	79	77	72	83	150	51	2	75	81	76

[a] X indicates that the inhaler was breathed but not assayed.

type inhaler and no regular (i.e., linear with concentration) dosage drops would be noted with the "solution" type inhaler if the vapor pressure were the controlling factor.

Unfortunately (from the standpoint of not having a more straightforward system to analyze), equilibrium and rate concepts are inextricably intertwined in the present situation. This easily leads to the basically incorrect tendency to try to predict kinetic data from thermodynamic values. However, because vaporization is relatively unencumbered with exotic entropy and orientation factors, rates of volatilization are often qualitatively proportional to the equilibrium properties of the materials involved. For example, equimolar quantities of the following compounds, allowed to evaporate at room temperature under the same conditions, will complete the evaporation process in this order: ether, acetone, chloroform, carbon tetrachloride, ethyl acetate, water. This order corresponds to both the materials' vapor pressures and boiling points. To further becloud the cause-and-effect relationship, the very magnitude of the numbers is such that the partial vapor pressure of the phlorone increases proportionately with the mole fraction. The concentration of benzyl salicylate is 5.15 moles/L. in the solutions, whereas the phlorone concentrations are 0.08–0.63 moles/L. Thus, the situation takes the form of plotting X and Y when the relationship is $Y = X/K + X$; when K is much larger than X, X and Y are essentially proportional to each other, i.e., if one doubles, so does the other; when K is much smaller than X, Y may be virtually a constant through a whole range of X values.

Hence, while vapor pressure concepts should not be neglected entirely in inhaler development, it is the rates of volatilization which must be controlled or modified.

Synthesis of Some New Hydrazone Derivatives

By LAWRENCE J. FISCHER and BERNARD ECANOW

The synthesis of D-glucuronolactone and D-mannuronolactone *p*-cyanobenzoyl hydrazones is reported. Also reported is a new melting point for *p*-cyanobenzoyl hydrazide. Infrared studies supporting evidence that the hydrazones exist in the uronolactone and not in the uronic acid form is reported.

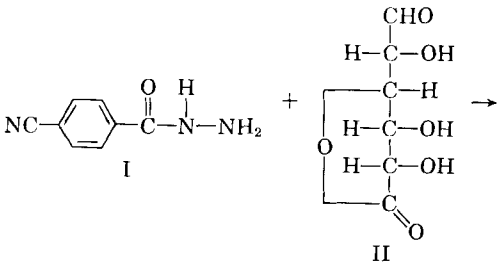
NUMEROUS derivatives of hydrazine have been prepared by Domagk, *et al.* (1), and tested along with other known derivatives for antitubercular activity. Among those compounds was the hydrazide of *p*-cyanobenzoic acid. This compound was reported to have significant antitubercular activity. The method of preparation of this compound has not been reported to date and is published here.

The synthesis was first attempted by reacting *p*-cyanobenzoyl chloride with 85% hydrazine hydrate or hydrazine sulfate. This method proved unsuccessful. The ethyl ester of *p*-cyanobenzoic acid was prepared and then reacted with 85% hydrazine hydrate in dioxane. This method was successful providing yields of up to 84% of *p*-cyanobenzoic acid hydrazide. If the reaction was allowed to proceed longer than 2 hours, the yield was reduced considerably with formation of substances melting above 300°. The solvent used for recrystallization was distilled water which yielded white crystals which melted at 202-203° (corrected). The reported melting point of *p*-cyanobenzoic acid hydrazide is 191°(1). A new melting point substantiated by analysis is now reported.

Uronolactone hydrazones of *p*-cyanobenzoic acid were prepared from the hydrazide in an effort to decrease the inherent toxicity of hydrazine derivatives. Sah has prepared uronic acid and uronolactone hydrazones of isonicotinic acid. These compounds were reported to be less toxic than isonicotinic acid hydrazide with no apparent loss of antitubercular activity (2).

The method of preparation of D-glucuronolactone and D-mannuronolactone *p*-cyanobenzoyl hydrazones followed the method of Sah (3) for the preparation of D-mannuronolactone and D-mannuronic acid isonicotinyl hydrazones. D-Glucuronolactone *p*-cyanobenzoyl hydrazone (III) was prepared by warming a solution of D-glucuronolactone (II) and *p*-cyanobenzoyl hydrazide (I) in absolute ethanol. The volume of the solvent was reduced and white crystals of the hydrazone crystallized from the solution.

In a similar procedure to that used for the preparation of D-glucuronolactone *p*-cyanobenzoyl hydrazone, D-mannuronolactone (V) and *p*-cyanobenzoyl hydrazide (IV) were dissolved in absolute ethanol. The final concentration of the reaction



I

II

III

IV

V

VI

Received November 9, 1961, from the College of Pharmacy, University of Illinois, Chicago Professional Colleges, Chicago.
Accepted for publication December 4, 1961.
Abstracted from a thesis submitted to the graduate faculty of the Chicago Professional Colleges of the University of Illinois by Lawrence J. Fischer in partial fulfillment of the requirements for the degree of Master of Science.

TABLE I.—URONOLACTONE *p*-CYANOBENZOYL HYDRAZONES AND *p*-CYANOBENZOIC ACID HYDRAZIDE

	Formula	Yield, %	M. p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
D-Glucuronolactone *p*-cyanobenzoyl hydrazone	$C_{14}H_{13}N_3O_6H_2O$	7.0.2	127 (decompn.)	49.86	49.91	4.48	4.53	12.46	12.20
D-Mannuronolactone *p*-cyanobenzoyl hydrazone	$C_{14}H_{13}N_3O_6$	80.8	169 (decompn.)	52.66	52.78	4.10	4.28	13.17	13.28
p-Cyanobenzoic acid hydrazide	$C_8H_7N_3O$	83.9	202–203	...	...	...	...	26.1	26.0

mixture was 0.001 mole of the reactants in approximately 220 ml. of absolute ethanol due to the relative insolubility of D-mannuronolactone in absolute ethanol. The product obtained from this dilute reaction mixture melted at 190–192°. Upon analysis, the compound was found not to be the hydrazone but possibly a product of the reaction of two moles of *p*-cyanobenzoic acid hydrazide. Regardless of the exact nature of the product formed here, it was not possible to obtain the hydrazone under such dilute reaction conditions.

As an alternate method, distilled water was used as a solvent for the reaction and an identical procedure followed. The white crystalline product obtained was analyzed to be D-mannuronolactone *p*-cyanobenzoyl hydrazone (VI) (See Table I).

EXPERIMENTAL

Infrared Data.—Infrared spectra were obtained using a Beckman IR-4 infrared recording spectrophotometer. All of the compounds prepared exhibited the characteristic cyano band in the range 2240-2210 cm.$^{-1}$. The spectra of D-glucuronolactone *p*-cyanobenzoyl hydrazone and D-mannuronolactone *p*-cyanobenzoyl hydrazone were found to be identical. This would seem to indicate the two compounds have identical structures except for the position of the hydroxyl on C_5, which apparently does not influence the infrared spectra. The spectra of the hydrazones contain a strong band of 1780–1760 cm.$^{-1}$, which is indicative of a C=O stretching vibration of a gamma lactone reported as 1780–1760 cm.$^{-1}$ by Bellamy (4). Supporting evidence that the hydrazones exist in the uronolactone and not the uronic acid form is the absence of the carboxylic acid OH stretching vibrations, which usually occur as broad bands with a series of minor peaks over the range 3000–2500 cm.$^{-1}$ (5).

Preparation of Uronolactone *p*-Cyanobenzoyl Hydrazones Ethyl *p*-Cyanobenzoate.—*p*-Cyanobenzoyl chloride (0.09 mole) was placed in a 100-ml. beaker and 10 ml. of absolute ethanol added. The mixture was heated gently on a steam bath and the reaction proceeded as evidenced by the formation of hydrochloride gas.

Upon completion of the reaction after approximately 10 minutes of gentle heating, the reaction mixture was neutralized with a solution of sodium bicarbonate. Upon neutralization, crystals of ethyl *p*-cyanobenzoate formed and were filtered from the solution. The product was recrystallized from ethanol and water; yield, 81.6%, m.p. 53–54° (lit. 54°).

***p*-Cyanobenzoic Acid Hydrazide.**—Three grams (0.017 mole) of ethyl *p*-cyanobenzoate was placed in a 100-ml. round-bottomed flask equipped with a reflux condenser and 25 ml. of *p*-dioxane added. Five milliliters of 85% hydrazine hydrate was added and the mixture refluxed for 1.5 hours. The reaction mixture was allowed to cool to room temperature and the solvent evaporated under a jet of air in an evaporating dish. The resulting crystals were dried at room temperature and recrystallized from hot distilled water.

D-Glucuronolactone *p*-Cyanobenzoyl Hydrazone.—*p*-Cyanobenzoic acid hydrazide, 5.0 Gm. (0.031 mole) was dissolved in 50 ml. of hot absolute ethanol. D-Glucuronolactone (Eastman), 5.3 Gm. (0.031 mole), was dissolved separately in 50 ml. of hot absolute ethanol. The solutions were mixed and heated to 50–60° on a steam bath for 15 minutes.

After cooling to room temperature, the solution was concentrated under a jet of air to 25 ml. It was placed in a freezer and white crystals formed within 1 hour. The white crystals were filtered from the solution and recrystallized from absolute ethanol.

D-Mannuronolactone *p*-Cyanobenzoyl Hydrazone.—*p*-Cyanobenzoic acid hydrazide, 0.5 Gm. (0.0031 mole) was dissolved in 75 ml. of hot distilled water. D-Mannuronolactone (Kelco No. 2065), 0.53 Gm. (0.0031 mole), was dissolved separately in 5 ml. of hot distilled water. The solutions were mixed and heated between 50 and 60° on a steam bath for 15 minutes. The solution was allowed to cool to room temperature, filtered, and placed in a refrigerator.

The white crystals which formed overnight were filtered from the solution and washed with cold distilled water. The product was recrystallized from distilled water.

SUMMARY

1. Two new hydrazone derivatives were prepared: D-glucuronolactone and D-mannuronolactone *p*-cyanobenzoyl hydrazones.

2. A new melting point for *p*-cyanobenzoyl hydrazide is reported.

3. Infrared studies supporting evidence that the hydrazones exist in the uronolactone and not in the uronic acid form is reported.

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Chemical Study of *Cestrum parqui*

By M. SILVA, P. MANCINELLI, and M. CHEUL

Solasonine and digitogenin were isolated and identified in the acid extract from *Cestrum parqui*.

THE BARK of *Cestrum parqui* L'Herit., a Chilean *Solanaceae*, is frequently used as an infusion for the treatment of common cold.

In connection with a systematic study on saponins of certain typical Chilean species (1–4), now under way in this laboratory, it appeared of interest to study *Cestrum parqui* in order to elucidate the presence or absence of alkaloids and sapogenins which have been reported repeatedly in current literature. Canham and Warren (5) have reported that *Cestrum parqui* contains two sapogenins, gitogenin and digitogenin. However, the authors failed to isolate any alkaloids. Mercier and Chevalier (6) had previously reported the presence of parquin. Several other investigators (7–10) had failed to isolate either parquin or any alkaloids.

This is a chemical study of the acetic acid extract of dried, pulverized, and benzene-defatted plant. This extract yielded a water-soluble fraction containing a saponin and a glycosidic alkaloid. The alkaloid was identified as solasonine. The saponin was hydrolyzed and its sapogenin identified as digitogenin. The benzene-soluble fraction yielded two products to be reported in a coming paper.

EXPERIMENTAL[1,2]

Isolation of Solasonine.—Stems and leaves of *Cestrum parqui* collected in April 1959 near Concepción, Chile, were dried at 80–90°. Nine kilos of the dried plant was ground and extracted in a Soxhlet extractor with benzene to exhaustion. The defatted plant material was dried at 80° and acid-soluble constituents were extracted with 5% acetic acid solution (11). This extract was then heated and precipitated with 5% ammonia solution. Subsequent filtering was extremely slow. The solid fraction was washed with diluted ammonia and dried at 90° to yield 184.5 Gm. The crude glycosidic alkaloid was partially purified by continuous extraction with ethanol.

The ethanol-soluble constituents were purified through repeated crystallization from ethanol-dioxane-water (4:4:2), yielding 31.5 mg. of solasonine; m.p. 260–264°. The infrared spectrum was identical to that of an authentic specimen.

Anal. (12).—Calcd. for $C_{45}H_{73}NO_{16}$: C, 61.1; H, 8.3; N, 1.6. Found: C, 61.2; H, 8.4; N, 1.6.

The melting point of the alkaloid was undepressed upon admixture with an authentic specimen, m.p. 264–266°.

Isolation of Digitogenin.—The alkaline filtrate from the precipitation of the alkaloid was concentrated and acidified with hydrochloric acid to render it approximately 2.5 *N*. The mixture was then refluxed for 9 hours and poured into water and ice. The precipitate was collected on a filter, washed with water, and dried to 90°; yield 195.3 Gm. The sapogenin was then extracted with petroleum benzin (b.p. 65–75°) in a Soxhlet extractor.

The extract was purified and recrystallized from ethanol to yield 8.55 Gm. of white needles, m.p. 264–267°. This product was chromatographed over 240 Gm. alumina Light H (13). Exhaustive elutions with benzene, chloroform, and ethanol yielded 31 fractions of 60 ml. each, which represented various degrees of purity of the same product. Paper chromatograms (14) of all eluted fractions indicated the presence of a single steroid. Fractions 8 to 18 were mixed to yield 355 mg. of digitogenin. This product was recrystallized from ethanol yielding white needles, m.p. 285–288°. This product did not show ultraviolet absorption and its infrared spectrum shows evidence indicating the presence of a steroid.

Anal.—Calcd. for $C_{27}H_{44}O_5$: C, 72.3; H, 9.9. Found: C, 72.0; H, 9.9.

Digitogenin acetate.—A solution of digitogenin (50 mg.) in pyridine (0.2 ml.) containing acetic anhydride (0.4 ml.) was boiled for 1 hour. The solid obtained by pouring the mixture into water was washed successively with 2 *N* hydrochloric acid, 5% sodium hydrogen carbonate solution, and water. Needles were obtained after recrystallization from ethanol; m.p. 233–235°.

Anal.—Calcd. for $C_{33}H_{50}O_8$: C, 69.0; H, 8.8. Found: C, 69.4; H, 9.1.

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Received August 7, 1961, from the Laboratorio de Fitoquímica, Departamento de Botánica, Instituto Central de Biología, Universidad de Concepción, Chile.
Accepted for publication November 5, 1961.
The authors wish to acknowledge the stimulation received from The British Council and the help of Mr. R. P. M. Bond for his ultraviolet and infrared analyses done at the Chemistry Department of The University of Glasgow. They are indebted to the Chemistry Department of the Massachusetts Institute of Technology for the analysis of solasonine, and to Dr. E. Bianchi of Universidad Católica de Chile for supplying an authentic specimen of alkaloid.

[1] Melting points are uncorrected.
[2] We are indebted to Dr. Alfred Bernhardt of the Max Planck Institut for the microanalysis of the samples.

Comparison Study of Alkyl Boronic Acids with Boric Acid

By STEPHEN M. SLEIGHT and MURIEL C. VINCENT

The bacteriostatic activities of two alkyl boronic acids were compared with boric acid. Some primary irritation and chronic toxicity determinations of the compounds were also accomplished. The results indicated that nonyl boronic acid and dodecyl boronic acid possessed bacteriostatic activity superior to boric acid, and that very dilute concentrations of these compounds could be employed to achieve bacteriostasis. Toxicological testing showed the alkyl boronic acids employed to be only mildly irritating and apparently nontoxic when applied to animal skin.

PRELIMINARY REPORTS from the manufacturer indicated that some of the alkyl boronic acids possessed a considerable degree of antibacterial activity in quite dilute concentrations. Comparison studies with the activity of boric acid were necessary to determine their possible value as substitutes for boric acid in existing types of preparations. It was also necessary to ascertain within certain limitations the possibility of the development of toxic reactions from their use.

The compounds employed in this study were nonyl boronic acid [nonyl dihydroxy borane, $C_9H_{19}B(OH)_2$] and dodecyl boronic acid [dodecyl dihydroxy borane, $C_{12}H_{25}B(OH)_2$]. They are colorless liquids, having about the same consistency as glycerin. Both are completely miscible at room temperature with hexane, benzene, ethyl ether, and acetone. Nonyl boronic acid is slightly soluble in water (3% by weight) and very soluble in methanol (34% by weight). Dodecyl boronic acid is less than 1% soluble in water and 23% soluble in methanol. The surface tensions of the saturated aqueous solutions at 25° are 47.2 and 44.9 dynes/cm.^{2}, respectively, for nonyl and dodecyl boronic acids. They are much weaker acids than boric acid (1).

EXPERIMENTAL

Determination of the Bacteriostatic Activity of the Alkyl Boronic Acids.—Determination of the bacteriostatic activity of nonyl and dodecyl boronic acid was made by use of the standard serum-cup agar plate method. Dilutions of 1:1,000, 1:5,000, 1:25,000 and 1:50,000 were prepared for both compounds. Table I illustrates the bacteriostatic activity of the various dilutions of these compounds against *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa*, and *Bacillus subtilis*.

Since there must be a basis of comparison to the presently used boric acid, the same tests were made with boric acid itself. The dilutions and organisms employed were identical to those employed with the alkyl boronic acids. The results of these tests are also indicated in Table I. At the dilutions used with the alkyl boronic acids, boric acid exhibited no detectable bacteriostatic activity. To obtain more positive values, dilutions of boric acid more commonly in use were prepared. These results are also given in Table I.

TABLE I.—BACTERIOSTATIC ACTIVITY

Compound	Dilution	Organism: *Micrococcus pyogenes* var. *aureus*	Organism: *Salmonella typhosa*	Organism: *Bacillus subtilis*
Nonyl boronic acid	1:1,000	4[a]	4	4
	1:5,000	2	3	3
	1:25,000	2	3	2
	1:50,000	1	2	2
Dodecyl boronic acid	1:1,000	4	4	4
	1:5,000	3	4	4
	1:25,000	3	4	3
	1:50,000	2	2	2
Boric acid	1:1,000	0	0	0
	1:5,000	0	0	0
	1:25,000	0	0	0
	1:50,000	0	0	0
Boric acid	5%	2	1	1
	2%	1	0	0
	1%	0	0	0

[a] 4, Complete bacteriostasis (zone of inhibition 0.7 cm.); 3, bacteriostasis not complete (zone of inhibition 0.5–0.7 cm.); 2, small zone of inhibition (less than 0.5 cm.); 1, very slight activity (zone of inhibition less than 0.5 cm., bacterial overgrowth); 0, no zone of inhibition (bacterial overgrowth).

The data obtained from this phase of the study would seem to indicate that the alkyl boronic acids possess a degree of bacteriostatic activity considerably superior to that of boric acid. There is, however, some possibility that the greater zones of inhibition achieved with the alkyl boronic acids may have been due in part to the relatively low surface tensions of these compounds in aqueous solution, allowing greater diffusion of the solution being tested into the surrounding media.

Determination of the Toxicity of the Alkyl Boronic Acids.—A primary irritation study was first accomplished according to the procedure outlined by Draize (2) and co-workers. This procedure involved the application of the compounds being tested to the intact and abraded skin of albino rabbits. A given compound was applied so that there were three applications to intact skin and three to abraded skin. At the end of a 24-hour exposure the resulting reactions were evaluated for erythema and edema formation. Evaluations were also made after 72 hours to determine the duration and intensity of any resulting irritation.

Table II illustrates the results of the irritation study as well as the method used to score the results. Averages of the 24- and 72-hour scores for all dilutions were calculated. The combined average figure obtained by this method is usually referred to as the primary irritation index.

The results of this phase of the study indicated that although the alkyl boronic acids were several

Received April 28, 1961, from North Dakota State University, College of Pharmacy, Fargo.
Accepted for publication June 22, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
Abstracted in part from a thesis presented by Stephen M. Sleight in partial fulfillment of the requirements for the degree of Master of Science.
The authors gratefully acknowledge Callery Chemical Co., Pittsburgh, for supplying the nonyl and dodecyl boronic acids used in this study.

TABLE II.—SCORES FOR PRIMARY IRRITATION

Compound	Rabbit Skin: Intact 24 hr.	Intact 72 hr.	Abraded 24 hr.	Abraded 72 hr.	Combined Average
Nonyl boronic	3[a]	1	3	2	
acid 1:1,000	1	0	2	0	
	2	1	2	1	
Average	1.33		1.67		1.50
Nonyl boronic	2	0	2	0	
acid 1:5,000	0	0	1	1	
	0	0	1	0	
Average	0.33		0.83		0.58
Nonyl boronic	0	1	1	1	
acid 1:25,000	0	0	0	1	
	0	0	0	0	
Average	0.17		0.50		0.33
Dodecyl boronic	4	2	6	3	
acid 1:1,000	2	3	4	4	
	2	0	2	1	
Average	2.16		3.33		2.74
Dodecyl boronic	2	1	3	2	
acid 1:5,000	1	1	2	1	
	1	0	3	2	
Average	1.0		2.17		1.58
Dodecyl boronic	0	0	1	0	
acid 1:25,000	1	0	2	1	
	0	1	2	0	
Average	0.33		1.0		0.67
Boric acid, 5%	0	0	0	0	
	0	0	1	0	
	1	0	1	0	
Average	0.17		0.33		0.25
Boric acid, 2%	0	0	1	0	
	0	0	0	0	
	0	0	0	0	
Average	0.0		0.17		0.08

[a] Evaluation of skin reactions:

Erythema formation
1, very slight
2, well defined
3, moderate to severe
4, severe
Total possible erythema score 4

Edema formation
1, very slight
2, slight
3, moderate
4, severe
Total possible edema score 4

Total possible score for primary irritation 8.

times more irritating than boric acid, they would still be classified as only mildly to moderately irritating to the skin.

A 20-day subacute toxicity determination was also performed with these compounds using the procedure outlined by Draize (3). The compounds and dilutions employed were the same as those used in the primary irritation study. In addition, a control was employed using normal saline solution. Quantities of 10 ml. per Kg. of body weight were applied daily to the clipped skins of albino rabbits. Each application covered approximately 10% of the body surface of the animal. The test animals were observed daily for visible symptoms of boron intoxication, and their body weights and food consumption were also checked daily. Weekly urine samples were collected and tested for boron content. The animals were also observed for a 2-week period following the last application for visible symptoms (4).

None of the animals died during the testing period. The two animals exposed to the lowest dilutions (1:1,000) of nonyl and dodecyl boronic acid and the control animal were sacrificed at the end of the observation period to be examined for pathological findings. Examination revealed no gross or microscopic evidence of boron poisoning in liver, kidney, or other tissues.

The results obtained by this study, while encouraging, should be extended to consider acute toxicity of these compounds by injection or oral ingestion.

SUMMARY

A comparison of the bacteriostatic activity, toxicity, and primary irritation of nonyl and dodecyl boronic acids with boric acid has been made.

The conclusions drawn from this study indicate that nonyl and dodecyl boronic acids possess bacteriostatic activity superior to the activity of the free form of boric acid. Very dilute concentrations of the alkyl boronic acids can be employed to achieve bacteriostasis.

Toxicological testing indicates that the alkyl boronic acids employed are only mildly irritating and apparently nontoxic when applied to rabbit skin. These results suggest that the compounds might be used as substitutes for ordinary boric acid in presently used topical preparations. *In vivo* testing of a more comprehensive nature is needed to establish the toxicity levels of these compounds by accidental ingestion or injection.

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Presence of 6-Methoxybenzoxazolinone in Uninjured Corn Tissue

Sir:

Wahlroos and Virtanen (1) have reported the isolation of a glucoside from rye seedlings which, after enzymatic transformation to the aglucone, is capable of being converted to benzoxazolinone. Virtanen finds that aglucone formation occurs when the rye seedlings are crushed. He stated that benzoxazolinone and 6-methoxybenzoxazolinone are not originally present in the rye, wheat, or maize plants, but arise only after the plant tissue is injured (2). In a subsequent paper Wahlroos and Virtanen (3), who used maize seedlings, isolated the glucosidic precursor to 6-methoxybenzoxazolinone by placing the intact seedling in boiling water in order to destroy the glycosidase.

On the basis of the above reports, it appeared that 6-methoxybenzoxazolinone (resistance factor A, RFA) which had been isolated in these laboratories (4) would only arise when corn tissue was injured. The following experiments were conducted in order to prove or disprove the presence of 6-methoxybenzoxazolinone in uninjured corn tissue.

Six young, high-resistance corn plants, 12 inches high, were cut and immediately immersed in liquid air. The frozen plants were powdered readily on placing them between two boards and applying pressure. The powdered material was immediately added to 2,500 ml. of 95% ethanol. Six more plants were cut at the same time and were allowed to stand at room temperature for 8 hours after being cut into small pieces.

Virtanen and co-workers found it necessary to hydrolyze the glucoside precursor to 6-methoxybenzoxazolinone by boiling the tissue for 2 hours in 2 *N* hydrochloric acid to effect *in vitro* cleavage to the aglucone, since the glucoside does not hydrolyze in boiling water alone. Plant material from the liquid air treatment and that obtained by our usual procedure were dried separately at 40–45° and extracted with ether in Soxhlet extractors for 48 hours. The ether solutions were concentrated, and an amount equivalent to 1.5 Gm. of fresh plant material was chromatographed (5).

The absorption maxima (228 and 285 mμ) and the extinction coefficients of the material eluted from the columns with 80:20 ether-ethanol were superimposable. The maxima were identical with those of authentic 6-methoxybenzoxazolinone.

Since no enzymatic or *in vitro* hydrolysis of the glucoside could take place in the tissue treated with liquid air and ethanol, it must be concluded, contrary to Virtanen's reports, that 6-methoxybenzoxazolinone does exist in uninjured corn tissue.

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Received December 29, 1961.
Accepted for publication January 9, 1962.
This study was supported in part by a research grant from the National Institutes of Health of the U. S. Public Health Service.

Method of Preparation of Soluble Radiophosphate

Sir:

The radiopharmaceutical, sodium radiophosphate solution,[1] obtained from commercial sources by this laboratory, showed, on many occasions, a loss of activity on filtration through Whatman No. 1 filter paper or on transferring to a different container owing to surface adsorption, even at the recommended pH range from 5.0 to 6.0 (1). These adsorbable radiophosphate preparations exhibited a different tissue distribution from that of a soluble radiophosphate when injected intramuscularly into the rat (2). Inasmuch as the radiological and therapeutic efficacy of this radiopharmaceutical depends upon its ready diffusibility and ionizability in solution near pH 7.0 (3), a method for the conversion of the adsorbable preparations into soluble radiophosphate would appear to be desirable.

Radiophosphorus (P^{32}) is prepared from sulfur by an (n,p) reaction. Following oxidation of the elemental radiophosphorus to radiophosphate, two methods are in current use for the isolation of the radiophosphate: (*a*) distillation of the bulk of the sulfur in a nitrogen atmosphere at 500° (4) and (*b*) addition of an nonisotopic carrier to transfer the radioactivity as a metal phosphate, followed by removal of the metal ions with resin column (5). The former method of preparation is used at the Chalk River Plant of Atomic Energy of Canada Ltd., and in our experience yields a soluble phosphate; the latter method is employed by the Oak Ridge National Laboratory and may give preparations unsatisfactory for reasons stated above.[2]

It has been suggested that the precipitate formed on neutralization of the solution of the unsatisfactory preparations is organic in nature (6). However, we have calcined the precipitate at 500° and digested it with aqua regia and were still unable to effect solution at pH 7.0. We have found, however, that a soluble radiophosphate can always be prepared from the unsatisfactory preparations by passing a 0.1 *N* hydrochloric acid solution of the phosphate through a Dowex 50-X8, 100–200 mesh resin column (hydrogen form, 1 cm. × 15 cm.) at a flow rate of approximately 2 ml./min. After the effluent is evaporated to dryness on a water bath, the residue is present as phosphoric acid in trace amounts. It is taken up in isotonic saline and the solution filtered to give the soluble radiophosphate.

Extensive conditioning of the cation exchange resin is essential for the success of the above procedure; this can be done with alternate 1 *N* hydrochloric acid and 1 *N* sodium hydroxide washes, followed by exhaustive extraction with 95% ethanol to remove any organic impurities. In order to minimize radiation damage to the resin, the carrier-free radiophosphate solution is diluted to give an activity of approximately 1 mc. per ml. for passage through the column. The resin column is washed with 0.1 *N* hydrochloric acid after use and stored. The overall loss of radioactivity due to manipulation is negligible.

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Received November 6, 1961.
Accepted for publication January 22, 1962.

[1] These commercial preparations were marketed as sodium radiophosphate solution U.S.P. and presumably conformed to all the U.S.P. specifications and cautions.

[2] This statement should not be interpreted to be a criticism of the quality of the product since the Oak Ridge National Laboratory has specifically indicated that "the product is not pharmaceutically refined" on form X-375 accompanying each radioisotope shipment.

Unusual Dissolution Behavior Due to Film Formation

Sir:

We have recently studied the dissolution of salicylates in basic aqueous media employing a procedure described previously (1). Dissolution ordinarily takes place at a constant rate under the experimental conditions referred to, since surface area and concentration gradient across the diffusion layer remain essentially constant. Thus, a plot of amount of substance dissolved *vs.* time is linear as shown in the dissolution of acetylsalicylic acid (ASA) and aluminum acetylsalicylate (Al-ASA) in 0.1 *N* hydrochloric acid (1). While a similar linear relationship is obtained in the dissolution of ASA in basic aqueous media, the dissolution rate of Al-ASA does not remain constant but decreases with time until it becomes very low. This decrease in dissolution rate of Al-ASA is due to the formation of a basic, water-insoluble aluminum compound on the surface of the drug solids. Addition of EDTA to the dissolution medium prevents the formation of such a film by chelation of Al^{+++} and, consequently, the dissolution rate in this medium remains constant under the experimental conditions. The weight loss of an Al-ASA pellet after partial dissolution in a basic aqueous medium containing EDTA is consistent with the amount of salicylate found in solution. In the absence of EDTA, the weight loss of an Al-ASA pellet is less than the theoretical weight loss (as calculated on the basis of the amount of salicylate in solution), the added weight being due to the deposited layer of the basic aluminum compound. A more detailed discussion of this phenomenon, its mathematics, as well as a description of the effect of variables such as pH, type and capacity of buffer, and stirring rate will be included in a future publication.

Our observations may have biopharmaceutical implications as indicated by a consideration of the gastrointestinal absorption pattern and dissolution behavior of Al-ASA. Salicylate given in the form of this drug is much more slowly absorbed than when given as ASA and is, in fact, not fully available to humans under the experimental conditions of a study described in a previous report from this laboratory (1). Despite the slow and incomplete absorption of salicylate after Al-ASA administration, the absorption peak occurred at the same time as after ASA administration, instead of being delayed as would be expected. The slower absorption of salicylate from Al-ASA can be explained on the basis of the slower dissolution of this drug, as compared with ASA, but the relatively early absorption peak cannot. The latter is apparently due to the deposition of the described film on Al-ASA solids in the small intestine. Thus, drug dissolution and absorption occur at a maximum rate during the early post-ingestion period when Al-ASA is in an acidic environment where no film formation takes place.

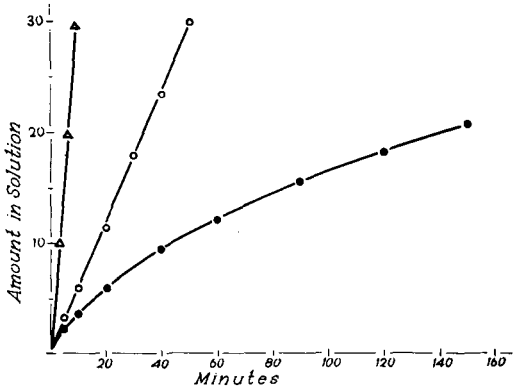


Fig. 1.—Dissolution of ASA and Al-ASA in 0.1 *M* Tris buffer of pH 8.0. △ ASA, ● Al-ASA, ○ Al-ASA with 1% EDTA added to dissolution medium. (Amount in solution expressed as mg. of salicylic acid.)

Film formation as a factor which may affect drug dissolution and absorption has, to our knowledge, not been described previously and is reported at this time in view of its biopharmaceutical implications.

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Received December 26, 1961.
Accepted for publication January 5, 1962.

Book Notices

Synthetic Methods of Organic Chemistry. Vol. 15. By W. THEILHEIMER. Interscience Publishers Inc., 250 Fifth Ave., New York 1, N. Y., 1961. xvi + 68 pp. 15 × 22.5 cm. Price $46.75.

New and improved methods of synthesis are recorded continuously in this series of books. This is the final volume of the third series and it includes a cumulative index to volumes 11–15. The body of the text carries all the reaction titles of volumes 11–15. Methods are given in abstract form. A valuable reference work.

Pharmakologie. 4th ed. By KNUD O. MØLLER. Benno Schwabe & Co., Verlag, Basel, Switzerland. Sole representatives in U. S. and Canada, Intercontinental Medical Book Corp., 381 Park Ave. South, New York 16, N. Y., 1961. 916 pp. 16.5 × 24 cm. Price $17.

Widely used in the Scandinavian and other European countries, this book (in German) has been revised to include many new drugs. A good index enhances its value as a reference textbook.

Rogers' Inorganic Pharmaceutical Chemistry. 7th ed. By TAITO O. SOINE and CHARLES O. WILSON. Lea & Febiger, Washington Square, Philadelphia 6, Pa., 1961. 704 pp. 15.5 × 23.5 cm. Price $10.

Inorganic chemicals used in medicine and pharmacy are covered extensively in this edition of the widely used textbook. Useful material from U.S.P. XVI and N.F. XI has been incorporated. A good textbook and reference volume.

Histopathologic Effects of Local Anesthetic Drugs and Related Substances. By PHILIP PIZZOLATO and WALTER MANNHEIMER. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1961. vii + 100 pp. 15 × 22.5 cm. Price $5.50.

Drugs which may produce local injury as they penetrate skin, subcutaneous tissues, muscles, and nerves are discussed. Many photographs illustrate the brief text.

Basic Biochemistry. By M. W. NEIL. Medical Dept. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1961. xii + 360 pp. 14 × 21.5 cm. Price $6.75.

The immediate relevance of some of the current biochemical knowledge is stressed in this preclinical textbook, but it also includes material which indicates the potentiality and scope of biochemistry.

Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest. By H. GOBIND KHORANA. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1961. x + 141 pp. 15 × 22.5 cm. Price $5.25.

This book is based on lectures given in 1959 on some aspects of the chemistry of phosphate esters; particularly nucleotides and related compounds. Special emphasis was given to the recent (up to 1959) synthetic work.

Pharmacognosy. 4th ed. By EDWARD P. CLAUS. Lea & Febiger, Washington Square, Philadelphia 6, Pa., 1961. 565 pp. 17.5 × 25.5 cm. Price $12.50.

The editor has incorporated the latest available information into his biochemically classified, monograph arrangement of drugs. This revision includes U.S.P. XVI and N.F. XI material. A good index makes the book useful for reference as well as a teaching text.

Modern Pharmacology and Therapeutics. 2nd ed. By RUTH D. MUSSER and JOSEPH G. BIRD. The Macmillan Co., 60 Fifth Ave., New York 11, N. Y., 1961. xiv + 889 pp. 14 × 21 cm.

A textbook intended primarily for nurses, the second edition presents the principles of drug therapy and its practice. It includes most of the drugs that were new at the time of its revision. The text is clear and concise, and a good index is appended.

Problems of Pulmonary Circulation. Edited by A. V. S. DE REUCK and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston, Mass., 1961. viii + 96 pp. 12 × 18.5 cm. Price $2.50.

Six reports and discussions related to the general title, which were given at the Ciba Foundation study group No. 8, are included in this pocket size booklet. The meeting was held November 1960. A subject index is appended.

The Pfizer Handbook of Microbial Metabolites. By MAX W. MILLER. The Blakiston Division, McGraw-Hill Book Co., 330 W. 42nd St., New York 36, N. Y., 1961. x +772 pp. 15 × 23 cm. Price $15.

Basic data on microbial metabolites are compiled in this book. Also included are current information and theories on biosynthetic routes to microbial metabolites. Three separate indexes list compounds (metabolites) by chemical name, by empirical formula, or by producing organism. Over 1,300 metabolites are listed; products of the *Streptomyces* predominating. The book is a new and useful reference source.

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Separation of Heavy Metals. By A. K. DE. Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1961. 308 pp. 15.5 × 23 cm. Price $9.

Applications of liquid-liquid extraction and ion-exchange techniques to the separation of heavy metals if Groups I–VIII are given in the first two sections of this book. Analytical procedures are given in the third part of the test.

Phosphorus and Its Compounds. Vol. 2, Technology, Biological Functions, and Applications. Edited by JOHN R. VAN WAZER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y. xvi + 1,092 pp. 15 × 23 cm. Price $35.

The second volume of this treatise is intended for industrial and academic researchers and also for business administrators, sales and purchasing personnel, inventors, and technologists. Volume I covered the modern chemistry of phosphorus and its compounds. Patented products utilizing phosphates are given by number and are briefly abstracted in appendixes covering 50 pages. A general index is included.

Invitation to Chemical Research. By E. EMMET REID. Franklin Publishing Co., Inc., Palisade, N. J., 1961. xviii + 366 pp. 14 × 21 cm. Price $10.60.

A noted research scientist and inspiring teacher sets out guide lines for the enticement and introduction of the novice to chemical research. Many advanced researchers might benefit from a reading of this book. A general index is appended.

Antimicrobial Agents Annual 1960. Edited by PETER GRAY, BENJAMIN TABENKIN, and S. D. BRADLEY. Plenum Press, 227 West 17th St., New York 11, N. Y., 1961. xviii + 670 pp. 16 × 25 cm. Price $8.50 domestic, $10 foreign.

The more than 90 papers and pertinent comments reported at the Conference on Antimicrobial Agents sponsored by the Society for Industrial Microbiology and held October 26–28, 1960, in Washington, D. C., are compiled in this volume. It includes the latest information publicly available in this field at the time of publication and is, therefore, a very valuable reference source. All the papers and panel discussions are listed in the Table of Contents but a general index is not included. The general subject areas covered are: New products, Modes of action, Laboratory studies, Antifungal agents, Clinical studies, Antitumor agents, and other related topics.

Alkaloid-Bearing Plants and Their Contained Alkaloids. Technical Bulletin No. 1234. Agricultural Research Service, U. S. Dept. of Agriculture. Superintendent of Documents, Government Printing Office, Washington 25, D. C. 287 pp. 15 × 23 cm. Paperbound. Price $1.

A useful reference and cross-reference of alkaloids and the plants in which they occur. The tabulated entries include 3,671 plants, some containing many alkaloids. The most common alkaloid appears to be lycorine (narcissine) which is listed as a constituent in 85 different plants.

Russian-English Chemical Dictionary. 1st. ed. By EUGENE A. CARPOVICH. Technical Dictionaries Co., Box 144, New York 31, N. Y., 1961. 352 pp. 14 × 21.5 cm. Price $14.

Terms used in chemistry, physical chemistry, chemical engineering, pharmacology, and foods are included in this dictionary. The English words generally are given the spelling common to the U. S. and the "c" in ergot alcaloids is only a typographical error. A knowledge of at least the Russian alphabet is required; there is no English-Russian section.

Accepted Dental Remedies 1962. 27th ed. Council on Dental Therapeutics of the American Dental Association. American Dental Association, 222 E. Superior St., Chicago 11, Ill., 1962. xiv + 235 pp. 15.5 × 20.5 cm. Paperbound. Price $3.

Dentists must have this latest edition of ADR in order to be up-to-date in their practice. The book contains so much more than the "accepted" drugs that it might be subtitled "The Dentist's Therapeutic Handbook." Pharmacists also can find useful information in this concisely and clearly written volume.

The revised edition contains expanded sections on dental therapeutics. The monographs have been reviewed by the Council and its consultants and have been rewritten in the light of current scientific information.

"Accepted Dental Remedies 1962" contains 249 pages, including the provisions for acceptance of products, a general index, a distributors' index, an index of current reports from the Council and the Division of Chemistry, and an index of more recent reports on products not listed in the book, including those classified in Groups B, C and D.

The Council on Dental Therapeutics has again performed its task of revision in an exemplary manner.

An Algorithm for Translating Chemical Names to Molecular Formulas. By EUGENE GARFIELD. Institute for Scientific Information, 33 South 17th St., Philadelphia 3, Pa., 1961. ix + 68 pp. 21.5 × 28 cm. Paperbound. Price $5.

A doctorate dissertation which develops a procedure for converting chemical names to formulas. The term algorithm appears to be used synonymously with procedure; and it is defined (Webster's dictionary) as a procedure for calculating with symbols.

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Gynecologic Endocrinology. By EDWARD A. GRABER. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1961. xiv + 218 pp. 15 × 22.5 cm. Price $7.50.

Designed as a practical guide for clinical diagnosis of glandular pathology, the book is a concise reference manual for the physician. The final section (X) of the book lists commonly used marketed hormone preparations and includes a table of comparative potencies. A general index is appended.

Separation of Heavy Metals. By A. K. DE. Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1961. 308 pp. 15.5 × 23 cm. Price $9.

Applications of liquid-liquid extraction and ion-exchange techniques to the separation of heavy metals if Groups I–VIII are given in the first two sections of this book. Analytical procedures are given in the third part of the test.

Phosphorus and Its Compounds. Vol. 2, Technology, Biological Functions, and Applications. Edited by JOHN R. VAN WAZER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y. xvi + 1,092 pp. 15 × 23 cm. Price $35.

The second volume of this treatise is intended for industrial and academic researchers and also for business administrators, sales and purchasing personnel, inventors, and technologists. Volume I covered the modern chemistry of phosphorus and its compounds. Patented products utilizing phosphates are given by number and are briefly abstracted in appendixes covering 50 pages. A general index is included.

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

April 1962 volume 51, number 4

Review Article

Pharmacology and Biochemistry of Drug Metabolism

By WILLIAM F. BOUSQUET

IN RECENT years, much emphasis has been placed on explaining drug action at the subcellular or biochemical level. In order that this be done in a meaningful and precise manner, it is necessary that such parameters as drug absorption, tissue distribution, elimination through excretory pathways, binding to constituents of cells and body fluids, and metabolism be thoroughly explored. Pharmacologists and biochemists appear agreed that the subcellular approach to drug action, whether from the enzymatic or physical biochemical points of attack, will yield a wealth of information concerning sites and mechanisms of drug activity. Metabolism of drugs is the parameter of prime concern in this review. The author will limit himself to a discussion of recent researches undertaken to define general metabolic pathways in which drug substances (and many other organic chemicals) participate. The reader should bear in mind that the related phenomena of drug absorption and elimination play an important role along with drug metabolism in the total spectrum of drug activity. Excellent reviews by Nelson (1), Wagner (2), and Butler (3) cover these topics.

The importance of drug metabolism as a contributing factor to the response noted with any drug *in vivo* is readily apparent upon consideration of the facts that (*a*) the duration of action of a drug can be related to the rate and manner in which it is metabolized, (*b*) the rate of elimination of a drug from the body is markedly dependent upon the physicochemical properties of its metabolic products, (*c*) the ability of a drug to penetrate cellular barriers and reach a site of action may be limited by its rate of biotransformation and the character of its metabolic products, (*d*) the toxicity of a drug can be reversed or, in certain cases, intensified upon conversion to a metabolite, and, (*e*) a metabolite may be formed *in vivo* which itself has a pharmacological effect ("active metabolite").

These points would indicate the importance of knowing the pathways of drug biotransformation as well as the degree of accumulation of metabolic products in tissue. It follows, too, that the toxicity and pharmacological effects of drug metabolites must be understood. From a more practical standpoint, recent legislation covering certain classes of physiologically active material (Food Additives Amendment) has made mandatory the identification and measurement of residual levels of drugs and drug metabolites which conceivably could gain entrance into the human food supply. Reviews on the subject of drug metabolism have appeared by Fishman (4, 135), Brodie, *et al.* (5, 6), and Maynert (7). The book, "Detoxication Mechanisms," by Williams (8) serves as an excellent source of information on the metabolism of drugs and other organic chemicals by providing encyclopedic treatment of the subject.

There is lack of agreement generally on the terms to be used in describing such studies. De-

Received from the Departments of Bionucleonics and Pharmacology, School of Pharmacy, Purdue University, Lafayette, Ind.

Supported in part by Grant A-4444 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

The author wishes to express his appreciation to Dr. John E. Christian, Bionucleonics Department, and Dr. Tom S. Miya, Pharmacology Department, for their suggestions and review of this paper.

toxication is obviously misleading since many drugs can be converted *in vivo* to more toxic compounds as exemplified by certain insecticides containing the —P=S grouping which is converted to the highly toxic —P=O form. This term has, nonetheless, withstood the test of time, and is currently favored by some authors. Others find fault with the use of "drug metabolism" in describing such work, preferring to retain the word metabolism to describe studies on endogenous substrates. The phrase, "drug biotransformation," attributable to Brodie, is perhaps the best terminology in such cases. The present author, however, prefers the use of "drug metabolism" and such will be employed in this review.

Although studies of the metabolic fate of drugs have been carried out since the end of the nineteenth century (9), it is only in the past decade that significant advances have been made concerning the enzymes involved. The excellent work of Axelrod, Brodie, and their co-workers (10–12) has established that the primary site of drug metabolizing activity in the mammal is in the microsomal fraction of the liver, necessary cofactors for the various reactions being found in the soluble fraction. It is of interest that the enzyme systems involved are apparently quite nonspecific in the sense that several reactions can be carried out by the same fraction. Specific enzymes have not been isolated to date. Nearly all of the classical reactions undergone by drugs *in vivo* including hydroxylation, dealkylation from amino nitrogen, ether cleavage, glucosiduronide formation, and reduction of the nitro and azo groupings have been duplicated under *in vitro* conditions using the mammalian liver microsomal system. Further, it has been established that the microsomal enzymes which act upon drugs and other foreign compounds appear unable to carry out the same reactions on endogenous substrates. Isolation and thorough understanding of the activity of the microsomal enzymes which metabolize drugs would seem to be the next step in the chain of events.

Biochemical Reactions Undergone By Drugs.—For purposes of discussion the more important metabolic pathways entered into by drugs can be classified into five groups. These five groups comprise some 15 distinct metabolic reactions, and cover the majority of the biotransformations which drugs and foreign compounds undergo. These are:

1. *Oxidation.*—Reactions considered under this heading are oxidations only in the sense that oxygen (as hydroxyl) takes part or is postulated to take part in one or more of the steps leading to the final metabolite, as proposed by Brodie, *et al.* (5): (*a*) hydroxylation of aromatic rings; (*b*) oxidation of aliphatic side chains to alcohols, ketones, or carboxyl groups; (*c*) dealkylation from oxygen (ether cleavage); (*d*) dealkylation from amino nitrogen; (*e*) deamination; (*f*) sulfoxide formation.

2. *Reduction.*—(*a*) Reduction of the nitro group, (*b*) cleavage and reduction of the azo linkage.

3. *Conjugation Processes.*—(*a*) Glucosiduronide formation, (*b*) formation of aryl sulfates, (*c*) conjugations with glycine, (*d*) acylation.

4. *Hydrolytic Reactions.*—(*a*) Cleavage of esters, (*b*) cleavage of amides.

5. *Exchange Reactions.*—(*a*) Replacement of sulfur by oxygen.

Table I indicates the character of each of these reactions and gives illustrative examples of drug substances known to undergo each of the biotransformations indicated.

Changes in Drug Properties through Metabolism.—Upon metabolism a drug is ordinarily converted to a more polar compound having a lipid solubility much less than that of the parent material. Current knowledge concerning the ability of organic compounds to pass tissue and cellular barriers indicates that it is the molecular form of the compound which is transported to the exclusion of the ionized species (13–15). Thus, the dissociation constant of the drug or metabolite and its inherent lipid solubility appear to be the primary factors governing its passage into cells. This is true whether one considers gut absorption (13), passage through barriers such as the blood-cerebrospinal fluid barrier (16, 17), or kidney elimination (18, 19). Thus it is likely that a drug having been converted to a more polar metabolite will, at physiological pH, be rather rapidly excreted, and will be unlikely to be returned to the tissues. This is indicated by recent studies in these laboratories on the metabolism of C-14 salicylamide and C-14 meprobamate. In neither case was it possible to demonstrate the presence of the relatively more polar metabolites of these drugs (salicylamide → 2,5-dihydroxybenzamide and glucosiduronides; meprobamate → hydroxymeprobamate and glucosiduronides) in body tissues of the rat (20, 21). Through metabolism, then, another factor is brought into play which contributes to termination of drug action, namely that the physical and chemical properties of the metabolite are such that it is unable to pass the lipid-like membranes between plasma and cells,

and it cannot readily be reabsorbed through the renal tubular epithelium.

Binding of drugs to proteins, particularly those of the plasma, can also play a role in determining the length of action of the drug, especially if therapeutic concentrations of free drug can be maintained from the storage depot. Liver, for example, shows a high capacity to bind mepacrine (22); antibiotics, sulfonamides, and certain barbiturates are bound in plasma to a significant degree. Through metabolism, one would expect this type of storage mechanism to be greatly altered. Storage of lipid-soluble drugs in body fat is frequently discussed as a contributing factor to the duration of drug action. This factor has been carefully studied in relation to certain of the thiobarbiturates (19, 23). A drug, upon being converted to a more polar metabolite, would be less lipid-soluble and less likely to be stored in fat depots.

Liver Enzymes Which Metabolize Drugs.— The report by Axelrod (10) who, in 1955, demonstrated that microsomes from rabbit liver could metabolize amphetamine to phenylacetone and ammonia was the first to demonstrate the localization of drug metabolizing activity. His studies of the cofactors involved indicated that the system required both reduced triphosphopyridine nucleotide (TPNH) and oxygen. Upon fractionation of whole liver into nuclei, mitochondria, microsomes, and soluble fraction, the microsomes alone could metabolize amphetamine, but at a very slow rate. Addition of the soluble fraction greatly increased the ability of microsomes to metabolize amphetamine. The soluble fraction containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase serves as a TPNH generating system. Washed rabbit liver microsomes could metabolize amphetamine equally well with added TPNH as with the soluble fraction. Axelrod also showed that the addition of unheated rat liver microsomes to the rabbit microsomes markedly decreased activity, whereas addition of heated rat microsomes did not, suggesting the presence of a heat labile inhibitor in the rat microsomes.

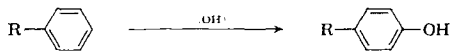
In vitro studies on drug metabolism are ordinarily carried out using microsomes isolated from liver homogenate by differential centrifugation, all necessary cofactors being added in the buffer medium. Some prefer the use of a fraction containing both the microsomes and the soluble fraction, prepared by centrifugation of the whole homogenate at 9,000 × g. Homogenates may be prepared either in isotonic potassium chloride or in 0.25 *M* sucrose, both yielding highly active preparations. General methods of homogenate preparation and fractionation are applicable (47–49). Table II shows the distribution of drug metabolizing activity in various subcellular fractions of rabbit liver. In general, only the liver yields active drug metabolizing preparations; of the reactions listed in Table I, only nitro reduction and glucosiduronide formation occur at other sites in the body. Nitro reductase activity is associated with kidney, heart, lung, and brain, as well as liver. This reaction, however, requires anaerobic conditions and thus differs from the general TPNH-O_2 requirement (35). Coliform bacteria in the intestine can also reduce nitro compounds (50).

Glucosiduronide formation can take place in the kidney cortex and the gastrointestinal tract of several species (51).

The fact that the liver microsomal system appears to act only on exogenous substrates is difficult to explain. The phylogenetic distribution of drug metabolizing activity shows this function to be restricted, in the main, to the mammal. Aquatic animals are unable to carry out the oxidative reactions enumerated in Table I. An explanation for this may lie in the fact that aquatic species have excretory mechanisms which allow rapid removal of unchanged drug from the body, so that metabolic defense mechanisms are unnecessary (52). Brodie (52) suggests the evolution of liver microsomal enzymes in terrestrial animals. In explanation of the fact that endogenous substrates are not attacked by these enzymes, Gaudette and Brodie (30) have studied the dealkylation of a series of foreign and endogenous alkylamines. They show that only those compounds exhibiting a high chloroform/water partition ratio at physiological pH are dealkylated by the microsomal system. It is of interest that the endogenous substrates studied exhibit extremely low partition and are not metabolized. This suggests that the microsomes are protected by a lipid barrier which is impermeable to polar compounds. Such a hypothesis deserves further exploration. Their interesting data are partially reproduced in Table III. McMahon (67) has, in the main, confirmed the observations of Gaudette and Brodie (30) cited above.

Cofactor requirements for the microsomal enzymes carrying out oxidative reactions are quite similar for hydroxylation, ether cleavage, dealkylation, and deamination. pH optima are also similar varying only slightly from about pH 7.0 to 8.5, depending on the particular substrate. Reduced TPN is a general requirement for all of these reactions, as is molecular oxygen. DPNH

TABLE I.—SOME COMMON METABOLIC REACTIONS UNDERGONE BY DRUGS

Pathway or Reaction	Schematically[b]	Drugs which Undergo This Reaction (Reference)
1. Oxidation[a]		
(*a*) Hydroxylation		Acetanilide (24, 11), Salicylic acid (25), Salicylamide 50
(*b*) Side chain oxidation	$R—CH_2CH_3 \xrightarrow{(OH)} R—CHOHCH_3$	Pentobarbital (26, 27)
(*c*) Ether cleavage	$R—O—CH_3 \xrightarrow{(OH)} R—OH + HCHO$	Morphine (28) Phenacetin (29)
(*d*) Dealkylation from amino nitrogen	$R—NH—CH_3 \xrightarrow{(OH)} R—NH_2 + HCHO$	Morphine, Meperidine (12), Monomethyl-4-aminoantipyrine (30), Methamphetamine (31)
(*e*) Deamination	$R—CH_2CHNH_2CH_3 \xrightarrow{(OH)} R—CH_2COCH_3 + NH_3$	Amphetamine, Ephedrine (10)
(*f*) Sulfoxide formation	$R—S—CH_3 \xrightarrow{OH)} R—SO—CH_3$	Chlorpromazine (32, 33, 34)
2. Reduction		
(*a*) Nitro reduction	$R—NO_2 \longrightarrow R—NH_2$	Chloramphenicol (35)
(*b*) Azo reduction	$R—N{=}N—R \longrightarrow 2\ R—NH_2$	Prontosil (36)
3. Conjugation		
(*a*) Glucosiduronide formation	$R—OH + UDPGA \xrightarrow{\text{transferase}} RO\text{-glucosiduronide}$	Salicylates (20, 37), Morphine (38), Meprobamate (21), Codeine (39)
(*b*) Aryl sulfate formation	$R—OH + PAPS \xrightarrow{\text{transferase}} RO—SO_2OH$	Salicylamide (40)
(*c*) Conjugation with glycine	$R—COOH + H_2NCH_2COOH \longrightarrow R—CONHCH_2COOH$	Benzoic acid (41)
(*d*) Acylation	$R—NH_2 + \text{Acetyl-CoA} \longrightarrow R—NHCOCH_3$	Sulfonamides (42)
4. Hydrolytic reactions		
(*a*) Cleavage of esters	$R—COOR' \xrightarrow{\text{esterase}} R—COOH + ROH$	Procaine (43)
(*b*) Cleavage of amides	$R—CONH_2 \xrightarrow{\text{amidase}} R—COOH + NH_3$	Hydroxybenzamide (44)
5. Exchange reactions		
(*a*) Replacement of S by O	$R—CS—R \longrightarrow R—CO—R$	Thiopental (45) Parathion (46)

[a] As proposed by Brodie, *et al.* (5); detailed mechanisms and cofactors for the reactions to be discussed later. [b] Abbreviations used in review are as follows: AMP, ADP, ATP for adenosine mono-, di-, and triphosphates; UDP for uridine diphosphate; UDP-G for uridine diphosphoglucose; UDPGA for uridine dihosphoglucuronic acid; TPN and TPNH for the oxidized and reduced forms of triphosphopyridine nucleotide; DPN and DPNH for the oxidized and reduced forms of diphosphopyridine nucleotide; G-6-PO_4 for glucose-6-phosphate; APS and PAPS for adenosine-5′-phosphosulfate and 3′-phosphoadenosine-5′-phosphosulfate; acetyl-CoA for acetyl coenzyme A; PP for pyrophosphate.

TABLE II.—INTRACELLULAR DISTRIBUTION OF ACETANILIDE HYDROXYLATING ACTIVITY[a]

Cellular Fractions	*p*-Acetylaminophenol Formed, μm.	% Relative Activity
Whole homogenate	1.14	100
Supernatant (9,000 × g)	1.14	100
100,000 × g	...	...
Microsomes	0.01	0.9
Soluble fraction	0.01	0.9
Soluble + microsomes	1.14	100

[a] From Mitoma, *et al.* (24). Fractions equivalent to 250 mg. of liver incubated with 10 μm. of acetanilide. Activity of whole homogenate taken as 100%. The 9,000 × g centrifugation removes unbroken cells, nuclei, and mitochondria, showing that these fractions possess no metabolizing activity.

TABLE III.—LIPID SOLUBILITY AND DEALKYLATION OF A NUMBER OF ALKYLAMINES BY LIVER MICROSOMES[a]

% Compound Extracted into Chloroform	Compound	Relative Activity
Foreign Materials		
>95	Monomethyl-4-aminoantipyrine	100
>95	Ephedrine	110
>95	Demerol	95
>95	Parpanit	80
>95	Methadone	50
>95	Caffeine	20
12	Theophylline	0
Endogenous Compounds		
<3	Adrenaline	0
<3	Sarcosine	0
<3	Choline	0
<3	Creatine	0

[a] Data from Gaudette and Brodie (30). Distribution of each compound between chloroform and pH 7.4 phosphate buffer determined. Microsomes from 1 Gm. rabbit liver incubated with 5 μm. of substrate. Activity of 100 equivalent to the dealkylation of 1 μm. of substrate per hour.

cannot replace TPNH in the oxidative reactions. Nicotinamide is frequently added to homogenates to protect TPN, and Mg^{++} is a required ion. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase are required to maintain TPN in the reduced state. That the drug metabolizing enzymes are not related to the cytochrome chain is indicated by the fact that cyanide does not influence the reactions. Gillette, *et al.* (53), have studied the role of TPNH and oxygen in the oxidative reaction. They show that microsomes contain a TPNH oxidizing system which gives rise to hydrogen peroxide. It is attractive to speculate that the hydrogen peroxide so produced serves as the source of "active hydroxyl" in the oxidative reactions. They further showed that only those tissues possessing significant TPNH oxidase activity could catalyze the dealkylation of monomethyl-4-aminoantipyrine, as shown in Table IV. Further work is required to explain fully this striking parallel.

Brodie (52) shows the requirement for TPNH more directly in a study of the oxidation of hexobarbital, as indicated in Table V.

TABLE IV.—TPNH OXIDATION AND MONOMETHYL-4-AMINOPYRINE DEALKYLATION BY MICROSOMES OF VARIOUS RABBIT TISSUES[a]

Tissue	TPNH Oxidation O.D. 340/5	4-Aminoantipyrine Formed, μm.
Liver	0.189	0.44
Lung	0.065	0.04
Kidney	0.047	0.02
Heart	0.028	0.03
Muscle	0.012	0.01
Brain	0.012	0.00

[a] Data from Gillette, *et al.* (53). TPNH oxidase activity measured spectrophotometrically. Microsomes from 1 Gm. of tissue incubated with 5 μm. of monomethyl-4-aminoantipyrine.

TABLE V.—REQUIREMENT FOR TPNH IN OXIDATION OF HEXOBARBITAL BY LIVER MICROSOMES[a]

Components	Hexobarbital Oxidized, μm.
Microsomes + 1.0 μm. TPN	0
Microsomes + 1.0 μm. TPN + 4 μm. G-6-PO_4	0
Microsomes + 1.0 μm. TPN + 4 μm. G-6-PO_4 + G-6-PO_4 dehydrogenase	0.30
Microsomes + TPNH	0.61
Microsomes + 0.5 ml. soluble fraction + 1 μm. TPN	0.57

[a] Data from Brodie (52).

Species, Sex, and Age Differences in Drug Metabolism.—It was mentioned that aquatic animals do not possess the enzymes necessary to metabolize drugs, this activity being restricted to the mammal. Within the mammalian group, wide quantitative differences are seen in relation to metabolizing activity, as well as some qualitative differences. Thus, amphetamine in rats and dogs is metabolized by hydroxylation of the aromatic ring, whereas in rabbits the compound is deaminated (10). Cats are not able to synthesize glucosiduronides (51).

There are well-known species differences in quantitative response to certain drugs (54, 55). In certain cases these differences are explainable on the basis of relative drug metabolizing activity, there being an inverse relationship between drug metabolizing ability and duration of pharmacological response. Thus, Quinn, *et al.* (55), have shown that the duration of action of hexobarbital is in inverse relationship to hexobarbital metabolism in several species. Their data are shown in Table VI.

Similar results were found in studies of the metabolism of antipyrine, amidopyrine, and aniline, and provide a sound basis for explaining differences in drug duration of action, sensitivity to various drugs, and toxicity.

TABLE VI.—SPECIES DIFFERENCE IN DURATION OF ACTION AND IN METABOLISM OF HEXOBARBITAL[a]

Species	Duration of Action, min.	Biological Half-Life, min.	Relative Enzyme Activity
Mouse	12 ± 8	19 ± 7	598 ± 184
Rabbit	49 ± 12	60 ± 11	196 ± 28
Rat	90 ± 15	140 ± 54	134 ± 51
Man	...	360	...

[a] Data from Quinn, *et al.* (55). Duration of action measured by sleeping time. Biological half-life determined by plotting plasma level *vs.* time at several intervals. Enzyme activity assessed by measuring mcg. of hexobarbital metabolized per gram of liver per hour. 100 mg./Kg. dose of hexobarbital in mouse, rabbit, and rat; 50 mg./Kg. in the dog.

Sex differences in drug metabolism appear to be restricted to the rat. Quinn, *et al.* (55), demonstrated that following a 100 mg./Kg. dose of hexobarbital, female rats sleep about four times as long as males, whereas their enzyme activity is about one-quarter that of the male. It is interesting that hexobarbital metabolizing activity can be significantly altered in rats by administration of testosterone to females and estradiol to males. Control male rats exhibited a hexobarbital metabolizing capacity of 682 mcg. of drug/Gm. of tissue/hr., whereas estradiol-treated males metabolize only 177 mcg./Gm./hr. Female controls metabolized 134 mcg./Gm./hr., whereas testosterone-treated females metabolized 543 mcg./Gm./hr. Sleeping times in these animals were altered to the same degree. Inscoe and Axelrod have recently demonstrated that glucosiduronide formation in rats is also markedly sex-dependent, male animals showing approximately four times the activity of females (56).

Fouts and Adamson (57) report that the newborn rabbit is essentially unable to carry out side chain oxidation, dealkylation, hydroxylation, and deamination. Enzyme activity appears at the second week of life, increasing to adult values at about four weeks. In an experiment to test the possibility of enzyme inhibitors being present in the livers of young animals, the addition of baby rabbit supernatant (9,000 × g) to adult supernatant caused an 82% reduction in the ability of the adult preparation to metabolize amphetamine. Jondorf, *et al.* (58), in a study of drug metabolism in newborn mice and guinea pigs, demonstrated that the newborn mouse is unable to carry out dealkylation, side chain oxidation, and ether cleavage. Enzyme activity appeared at 7 days and reached heightened values at 21 days. It is also of interest that sensitivity of mice to hexobarbital decreased as the animal's ability to metabolize the drug increased. Similar results were obtained in guinea pigs, and these young animals were also unable to synthesize glucosiduronides. These data are significant in that they provide a rational basis for explaining the heightened sensitivity of young animals to certain drugs, mainly central nervous system depressants, apart from differences in blood-brain barrier.

Physiological and Biochemical Control of Drug Metabolizing Enzymes.—Little is known about the extra-hepatic factors which control the activity of the microsomal enzymes. The observation (55, 56) that sex hormones can alter microsomal activity is apparently confined to the rat and does not seem to be of general significance. Remmer (59) has shown that adrenalectomy reduces the capacity of liver microsomes to oxidize barbiturates. Zauder (60) has reported that adrenalectomy in rats destroys the ability of liver slices to conjugate morphine. It is interesting to speculate on the role of the adrenal cortex (and of the pituitary gland) in regulating liver microsomal activity. It is well known, for example, that administration of corticoids increases protein synthesis (61, 62). It might therefore be expected that the adrenal cortical secretions regulate synthesis of microsomal enzymes, at least to some degree. Cochin and Sokoloff (63) have investigated the effects of L-thyroxin on the demethylation of morphine in mice and report that it depresses this function. Since the experiments were carried out in intact animals rather than those which had been thyroidectomized, it is not possible to make a definitive statement regarding thyroxin effects on drug metabolism. Conney and Garren (64) report that thyroxin stimulates the metabolism of zoxazolamine and decreases the metabolism of hexobarbital. In their work with zoxazolamine it is of interest that during the period of greatest zoxazolamine metabolism, the tissue level of G-6-PO_4 and G-6-PO_4 dehydrogenase were also markedly increased.

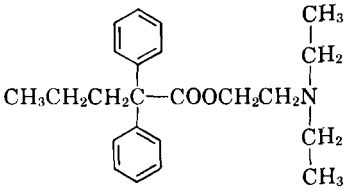
Recent studies by Dixon, *et al.* (65), demonstrate that the alloxan-diabetic rat shows a decreased ability to oxidize hexobarbital and chlorpromazine and to cleave the ether linkage in codeine. Administration of insulin to diabetic rats restores drug metabolizing activity to control values. A relationship is evident between glycogen levels in the liver and drug metabolizing activity, low levels of liver glycogen following alloxan paralleling depressed enzymatic function. McLuen and Fouts (66) show that livers from jaundiced rats possess a markedly decreased ability to carry out oxidation, deamination, ether cleavage, and dealkylation. They note that the effects seen in severely jaundiced animals are quite closely duplicated *in vitro* by the addition

of sodium deoxycholate ($10^{-3}M$) to tissue homogenates, and mention the possibility that effects seen in jaundiced rabbits may be due to increased concentrations of bile acids in the liver.

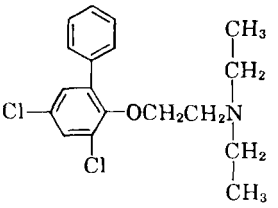
In 1954, Udenfriend, *et al.* (68), reported that a synthetic system consisting of ascorbic acid, ferrous sulfate, and EDTA in phosphate buffer and an oxygen atmosphere, was able to hydroxylate drugs and other foreign compounds to yield products identical to the hydroxylated metabolites of these drugs obtained *in vivo*. Thus, the hydroxylation of salicylic acid, acetanilide, aniline, and antipyrine was demonstrated (69). It has long been recognized that the administration of certain drugs can cause an increased excretion of ascorbic and glucuronic acids (70), which has been shown to be due to increased synthesis (71). Participation of ascorbic acid in certain drug metabolic reactions is thus suggested. Manthei (72) has shown that ascorbate-treated mice exhibit heightened tissue levels of isoniazid after dosing with this drug, and that a metabolite of isoniazid is recoverable in mice having the same chromatographic characteristics as a compound obtained by chemical reaction of isoniazid and dehydroascorbic acid. In an interesting study of the role of ascorbic acid in aromatic hydroxylation, Axelrod, *et al.* (73), studied the biological half-lives of acetanilide, aniline, and antipyrine in normal and scorbutic guinea pigs. They demonstrated that the biological half-lives of these drugs are significantly increased in the scorbutic animal. Ascorbic acid is known to participate, nonspecifically, in the biological oxidation of tyrosine (74). Before assigning a role to ascorbic acid in drug metabolism, the questions of specificity and detailed mechanism must be answered. For example, barbital, a drug which is not extensively metabolized, has a pronounced effect on stimulating ascorbic acid and glucuronic acid biosynthesis (75). A host of compounds, having different pharmacological effects and being metabolized along different pathways, also induce ascorbic acid synthesis (76). It is interesting to note that liver ascorbic acid is mainly concentrated in the microsomes and supernatant fractions, very little being found in the mitochondria and none in the nuclei (77).

The excellent work of Burns and his colleagues in providing us with details of the mechanism of ascorbic acid biosynthesis and its possible role in drug metabolism is deserving of mention (71, 75, 76, 78, 79).

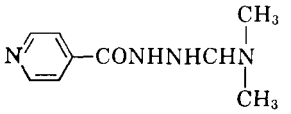
Drug Metabolism Inhibitors.—Macko, *et al.* (80), reported in 1953 that SKF 525-A (β-diethylaminoethyl diphenylpropylacetate, I) has the ability to potentiate the action of certain spinal cord depressants and central nervous system stimulants. Cook, *et al.* (81), in a detailed study, have shown that the duration of action of hexobarbital is significantly increased under the influence of SKF-525A. Other compounds shown to be potentiated by this agent include secobarbital, amobarbital, phenobarbital, and chloral hydrate (82); *d*-tubocurarine (83); and certain antiepileptic drugs (84). Other compounds known to exert similar effects on drug metabolism include Lilly 18,947 (2,4-dichloro-6-phenylphenoxyethyl diethylamine, II), iproniazid, III, and JB-516 (1-phenylisopropylhydrazine, IV).



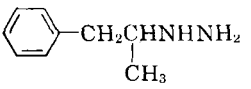
I



II



III



IV

SKF 525-A has been used extensively as a tool in drug metabolism studies. The mechanism of its action was explained first by Axelrod, *et al.* (85), who showed that it decreased the ability of the liver microsomes to carry out such reactions as side chain oxidation and demethylation and thus increased the duration of action of drugs being metabolized through these pathways. Axelrod, *et al.* (86), further demonstrated in a study of the effects of this compound on hexobarbital action that the increased duration of action was closely paralleled by an increase in the biological half-life of the drug. Of significance in this study was

the fact that upon return of the righting reflex in rats receiving 100 mg./Kg. hexobarbital, plasma levels of hexobarbital were no higher in the SKF 525-A-treated animals than in the control group. This would indicate that the increased sleeping time noted was not due to higher brain concentrations of the barbiturate. It has been shown (81) that administration of SKF 525-A after return of the righting reflex following hexobarbital treatment will not reinduce hypnosis.

In a study of the mechanism of SKF 525-A action, Cooper, *et al.* (87), demonstrated that the compound added to liver homogenates at concentrations of 10^{-4} *M* or greater can prevent oxidation of the barbiturates, hexobarbital, pentobarbital, and secobarbital; the demethylation of aminopyrine and meperidine; the deamination of amphetamine; ether cleavage of codeine, and the formation of morphine glucosiduronide. In order to test the possibility that the inhibition of drug metabolism seen with SKF 525-A might be due to its hydrolytic products, diethylaminoethanol and diphenylpropylacetic acid, *in vitro* experiments were run in which hexobarbital metabolism was studied under the effects of SKF 525-A and the hydrolytic products. Both SKF 525-A and diphenylpropylacetic acid were equally effective in preventing oxidation of the barbiturate, whereas diethylaminoethanol was without effect. SKF 525-A is considerably more active *in vivo* than is diphenylpropylacetic acid, presumably due to the fact that diphenylpropylacetic acid would be rapidly excreted. These authors examined the possibility that SKF 525-A might act by interfering with hydrogen transport from TPNH. They were able to show no effect of diphenylpropylacetic acid on the systems TPN-cytochrome c reductase, glucose-6-phosphate dehydrogenase, and isocitric dehydrogenase.

While the mechanism of action of this compound remains obscure, it has provided a useful tool in studies of drug metabolism in that certain inferences can be drawn about enzymes involved in various of the drug metabolic pathways by noting the quantitative effect of SKF 525-A on the pathway of interest. The possibility of several contributing factors to the mechanism of action of SKF 525-A must not be overlooked. For example, the effects of this compound on drug tissue distribution has not been extensively studied to date. Its ability to reduce the rate of excretion of S-35 pentothal has been reported by Achor and Geiling (88) and should be further investigated in relation to other compounds, quantitative studies being required of excretion rate *vs.* time-course of pharmacological response. The possibility of an interference by SKF 525-A in the function of a normal physiological or biochemical factor controlling the synthesis or function of the liver microsomal enzymes responsible for drug metabolism must not be overlooked.

Fouts and Brodie (89) have reported on the ability of compound Lilly 18,947 to block drug metabolic reactions. They show that this compound is about equally active with SKF 525-A in depressing demethylation, ether cleavage, deamination, and hydroxylation.

JB-516 has been shown by Pearce, *et al.* (90), to decrease the metabolism of hexobarbital in mice, presumably acting by a mechanism similar to that of SKF 525-A and Lilly 18,947. Welch, *et al.* (91), have demonstrated that chlorothion increases hexobarbital sleeping time in mice and, when added to liver slices, decreases the oxidation of the barbiturate. It is pointed out that both control and chlorothion-treated animals awaken when brain levels of hexobarbital are about 25 mcg./Gm. of tissue, again suggesting that receptor sensitization is not involved in the increased duration of pharmacological response.

Compounds Which Stimulate Drug Metabolism.—It was reported by Conney, *et al.* (92, 97), that pretreatment of rats with phenobarbital, barbital, 3-methylcholanthrene, or 3,4-benzpyrene markedly increases the activity of the liver enzymes azo N-demethylase, 3,4-benzpyrene hydroxylase, and zoxazolamine hydroxylase. The data can be confirmed by *in vivo* studies such as the fact that the duration of action of a paralytic dose of zoxazolamine can be shortened by pretreatment of the animals with 3-methylcholanthrene or 3,4-benzpyrene. It is of further interest that chronic administration of aminopyrine or phenylbutazone can increase activity of the liver microsomal enzymes responsible for their own metabolism. The possible implication of these findings in drug tolerance studies is apparent.

Conney, *et al.* (93), provide evidence that the increased activity of the liver microsomal system brought about by pretreatment of rats with phenobarbital is due to increased enzyme synthesis. They show that administration of ethionine, an amino acid antagonist, prevents the phenobarbital-induced increase in microsomal activity. Further, they point out that addition of phenobarbital to *in vitro* incubation mixtures is without effect in increasing the activity of azo N-demethylase activity, whereas administration

of the barbiturate to the intact animal yields homogenates with a substantially greater metabolic capacity. Such pretreated animals also exhibit increased total liver protein and increased liver microsomal protein. This evidence is consistent with a hypothesis of increased enzyme synthesis.

Cramer, *et al.* (94), have shown that the ability of liver homogenates or microsomes to hydroxylate the carcinogen 2-acetylaminofluorene is increased five to ten-fold in rats upon pretreatment with 3-methylcholanthrene. The effects seen with 3-methylcholanthrene were blocked when the animals were pretreated with *d,l*-ethionine. Von der Decken and Hultin (95) have confirmed the enzyme inductive effects of 3-methylcholanthrene in studies of the demethylation and reductive cleavage of *p*-monomethylaminoazobenzene, and the binding of 2-aminonaphthalene to soluble liver proteins. They show also that microsomes from 3-methylcholanthrene-treated rats have an increased capacity to incorporate leucine into protein and a higher RNA content, further substantiating the hypothesis of Conney, *et al.* (93), that increased drug metabolizing activity is due to increased enzyme synthesis.

It is interesting to note that some compounds which can elicit an increase in the activity of the liver microsomal enzymes metabolizing drugs, also markedly increase the biosynthesis and excretion of ascorbic acid (71, 76, 96).

Mechanisms of Specific Metabolic Reactions.—Of the metabolic reactions undergone by drugs listed in Table I, those which are of more general significance biochemically have received a great deal of study in recent years. The mechanisms of such general (and truly "detoxifying") reactions as glucosiduronide formation and aryl sulfate formation have been thoroughly elucidated.

Glucosiduronide Formation.—This reaction occurs with hydroxy compounds primarily, as well as with carboxylic acids and, in certain cases, with amines (N-glucosiduronides), and has been recognized and described for over 60 years (8). The occurrence of this reaction *in vivo* is now known to take place primarily in the liver, although kidney cortex and the gastrointestinal mucosa are also active (51, 98). Regardless of whether an ether type glucosiduronide (from a hydroxy compound) or an ester type glucosiduronide (from a carboxyl group) is formed, carbon atom 1 of glucuronic acid participates in the linkage. Williams (8) covers the mechanism of glucosiduronide formation in detail as well as presenting a historical account of this metabolic pathway. Dutton (99) has outlined the pathway of this reaction as follows

$$\text{glycogen} \xrightarrow{H_3PO_4} \alpha\text{-glucose-1-PO}_4 \quad \text{(Step 1)}$$

$$\alpha\text{-glucose-1-PO}_4 + \text{UTP} \xrightarrow[\text{transferase}]{\text{uridyl}} \text{UDP-glucose} + \text{PP} \quad \text{(Step 2)}$$

$$\text{UDP-glucose} \xrightarrow[\text{UDPG dehydrogenase}]{DPN,O_2} \text{UDP-glucuronic acid} \quad \text{(Step 3)}$$

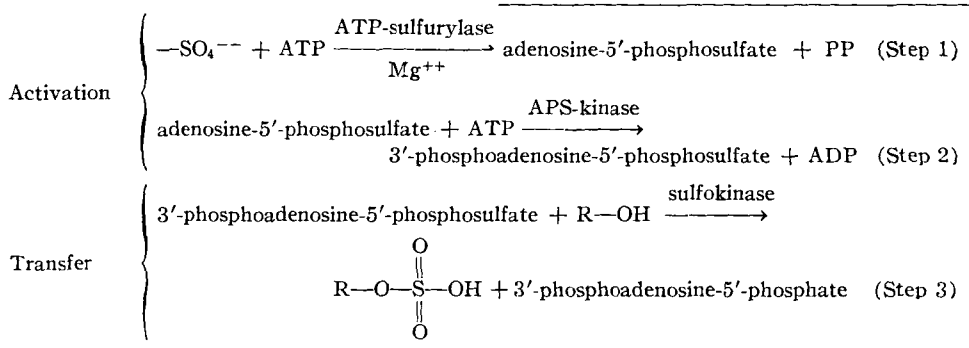
$$\text{UDP-glucuronic acid} + \text{R—OH} \xrightarrow[\text{glucuronyl transferase}]{} \text{R—O-glucuronic acid} + \text{UDP} \quad \text{(Step 4)}$$

That this pathway of metabolism is an important route leading to the elimination of drugs and other foreign compounds from the animal body is obvious; however, its role in normal biochemistry has not been well established. It is known, for example, that endogenous steroids are present in the blood as glucosiduronides (100) as well as being excreted in this form (101). Bilirubin is known to form such a conjugate (102). It has been postulated that steroids are "carried" in the blood as inactive conjugates being released by the enzyme β-glucuronidase which is widely distributed in mammalian tissues (103). The experimental and clinical significance of this is not clear.

Glucosiduronides, whether formed from foreign compounds or endogenous substrates, are rapidly hydrolyzed *in vitro* by the enzyme β-glucuronidase. This technique has proved useful in drug metabolism studies in providing a tool to confirm the suspicion that a conjugate of this type has been formed. The role which β-glucuronidase plays *in vivo* in nuclear. DeDuve (104) described the properties of a class of cytoplasmic particles found in liver, brain, and kidney, known as lysosomes, which appear to contain several enzymes including β-glucuronidase. It is stated that the particles are covered with a lipoprotein layer which is impermeable to the enzyme substrates. Enzyme activity can be released upon treatment of the particles with lecithinase, proteolytic enzymes, various detergents, or carbon tetrachloride. One questions the role which this "latent" β-glucuronidase might play in normal biochemistry.

Sulfate Conjugation.—This reaction represents another metabolic sequence of more general significance which is involved with the metabolism of a number of endogenous sub-

strates, as well as with a host of foreign compounds, including drugs. The so-called "ester sulfates" have been known since the work of Baumann in the last century (105), although the mechanism underlying the formation of such compounds has only recently come to light. Bernstein and McGilvery (106), and DeMeio, *et al.* (107), demonstrated that sulfate conjugation can occur in liver supernatants in the presence of adenylic acid, magnesium ion, and ATP. Lipmann (108) has reviewed developments on this problem, as have Bostrom and Vestermark (109). The generally accepted view for the mechanism of the synthesis is that it involves a two-step process, first, the activation of sulfate, and secondly, the transfer of sulfate to an acceptor molecule. The steps seem to be as follows

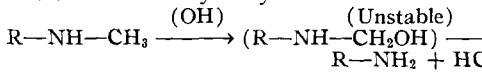


It is of interest that there appear to be at least three sulfate transferring enzymes (sulfokinases) which can take part in the last step of the sequence (108). One of these is called 3′-β-hydroxyl sulfokinase since it catalyzes sulfate transfer to steroids such as dihydroandrosterone, isoandrosterone, and progesterone which have a 3′-β-hydroxyl group. Another kinase appears to react with estrone forming estrone sulfate. The third is apparently quite nonspecific in that it can catalyze the transfer of active sulfate to a wide variety of phenolic acceptor substances. This is the enzyme of interest in drug metabolism studies. Of note in such studies is the suggestion by Anderton, *et al.* (110), that the pKa of the hydroxyl group should lie between 7 and 10 if the compound to which it is attached is to be conjugated with sulfate.

Little information is at hand relative to the role of sulfate conjugation in the normal animal. Certain end products of protein and steroid metabolism are excreted in the urine as sulfates, but the significance of this is not apparent. The lysosomes of DeDuve are stated to contain aryl sulfatase, an enzyme which can hydrolyze the ester sulfate linkage. Some properties of this enzyme have been reported by Webb and Morrow (111) and Roy (112). It appears to be activated by chloride ion (113).

Boyland (114) has reviewed other biochemical conjugations and points out the significance of such reactions as phosphate ester formation and mercapturic acid synthesis through glutathione.

Dealkylation.—The general scheme proposed for the removal of N-alkyl groups is that of Gaudette and Brodie (30) who postulate that "active hydroxyl" plays a role in the mechanism. This is consistent with the TPNH requirement and the formation of hydrogen peroxide during TPNH oxidation (53), which could serve as the source of "active hydroxyl"



Gaudette and Brodie (30) present data indicating that more than one enzyme system is active in the microsomes in the dealkylation reaction. Their data indicate that the removal of alkyl groups from 4-aminoantipyrine is dependent upon the nature of the alkyl group when varied from methyl to dibutyl, whereas dealkylation of the same groups from aniline is not so affected. Additionally, they show that SKF 525-A effectively inhibits the dealkylation of mono- and dimethyl-4-aminoantipyrine, ephedrine, and meperidine, but is without effect on the dealkylation of methyl-, ethyl-, and butylaniline, and the ethyl and butyl 4-aminoantipyrines. Further evidence for the presence of at least two enzymes responsible for dealkylation is indicated by the work of Conney, *et al.* (97), who show that the administration of 3,4-benzpyrene to rats causes an increase in microsomal enzyme activities toward 3-methyl-4-monomethylaminoazobenzene, N-methylaniline, and monomethyl-4-aminoantipyrine, whereas the dealkylation of meperidine and diphenhydramine is decreased. They point out that the mitochondrial system which can dealkylate endogenous substrates such as sarcosine differs from the microsomal system in that

it does not require TPNH and it is blocked by cyanide. The authors postulate that the system dealkylating sarcosine is a cytochrome linked dehydrogenase, the mechanism of the reaction being as follows

$$R{-}NH{-}CH_3 \xrightarrow{-2\,H} R{-}N{=}CH_2 \xrightarrow{HOH}$$
$$(R{-}NH{-}CH_2OH) \longrightarrow R{-}NH_2 + HCHO$$

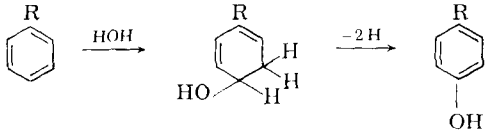
Other oxidative reactions such as sulfoxide formation, ether cleavage, and deamination are postulated to require "active hydroxyl" in sequences similar to that outlined for foreign N-alkylamines.

Aromatic Hydroxylation.—Several studies have confirmed that mammalian liver microsomes are the source of an enzyme(s) able to hydroxylate aromatic compounds (24, 25). TPNH and oxygen are requirements of the system as is the case with other oxidative reactions in the microsomes through which drugs are metabolized. The products of these hydroxylations may be phenols or, less commonly, dihydro diols in the case of condensed ring systems such as naphthalene.

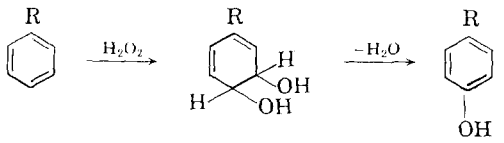
Posner, *et al.* (115), present evidence that more than one enzyme is involved in the hydroxylation mechanism. They cite experiments run with inhibitors showing that the hydroxylation of acetanilide to its 2- and 4-hydroxy derivatives is nearly completely inhibited by 2,2′-dipyridyl whereas the hydroxylation of quinoline is only slightly affected. Microsomes incubated with Tween 80 rapidly lose their ability to hydroxylate acetanilide and quinoline, while the hydroxylation of naphthalene to 1,2-dihydronaphthalene-1,2-diol is little affected.

Endogenous substrates such as steroids (116) and phenylalanine (117) are also hydroxylated. Phenylalanine hydroxylase, while present in a 25,000 × g supernatant of liver, appears to differ from the hydroxylases involved in the metabolism of foreign compounds in that DPNH serves much better than TPNH as a cofactor.

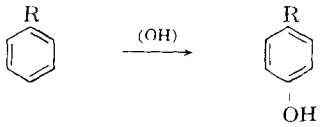
Three possible routes by which an aromatic ring may be hydroxylated have been proposed by Udenfriend, *et al.* (68), in discussion of their Fe^{++}-ascorbic acid system. (*a*) Addition of water to a double bond followed by dehydrogenation



(*b*) Reaction with hydrogen peroxide to form a dihydro-diol intermediate followed by loss of water



(*c*) Direct substitution of hydroxyl for hydrogen



Posner, *et al.* (118), have recently studied the mechanism of aromatic hydroxylation using rabbit liver microsomes incubated in media containing HTO, H_2O^{18}, and in the presence of O_2^{18} in order to determine distribution of hydrogen and oxygen in the metabolite. The conversion of acetanilide to 4-hydroxyacetanilide contains no O^{18} arising from H_2O^{18} which indicates that addition of water plays no role in the mechanism. Phenolic oxygen in the metabolite is derived solely from O_2^{18}. No incorporation of tritium from HTO was found in the 4-hydroxyacetanilide formed. These data do not rule out the possibility that hydrogen peroxide arising during the oxidation of TPNH might be involved in the mechanism as a source of molecular oxygen or "active hydroxyl."

Imai and Sato (119, 120), in an attempt to localize hydroxylases from liver microsomes, report that an acetone powder prepared from microsomes is almost completely unable to carry out hydroxylations, whereas addition of the lipid-rich acetone-soluble fraction restores this function.

Formation of Active Metabolites.—There is now much data in the pharmacological literature suggesting that metabolites of drugs can contribute significantly to total drug response. This is to be expected and in no way conflicts with the general rule that drug metabolites are more rapidly excreted than the parent compound. This possibility should not be overlooked in studies of the metabolic products of any new drug, and is undoubtedly of significance with many older compounds in therapy which have not been fully investigated in the light of recent advances in methodology and understanding of drug metabolism in general.

Thus, the conversion of the barbiturate thiamylal, 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid, to the active metabolite, secobarbital, was demonstrated *in vivo* by Spector and

Shideman (121), as well as the conversion of the thiobarbiturate, thiopental, to the active species, pentobarbital through exchange of sulfur and oxygen (122). An excellent paper by Butler and Mahaffee (123) shows that the activity of the antiepileptic drug, trimethadione, is in fact due to its demethylated metabolite. Fishman (4) cites several other examples in the barbiturate and anticonvulsants groups in which active metabolites are formed.

It has long been recognized that acetylsalicylic and salicylic acids are converted *in vivo* to the hydroxylated metabolite, gentisic acid (124, 125, 126). This compound also exhibits analgetic properties and reduces fever (127, 128). Conversion of the salicylamide molecule to gentisic acid amide gives rise to a compound exhibiting antihistaminic properties (129), whereas salicylamide itself is devoid of this effect.

Levi and Snow (130) present data showing the *in vivo* conversion of bis-*p*-aminophenylsulfoxide to the corresponding sulfone which has antileprosy properties. Conversion of acetophenetidin to the active analgetic N-acetyl-*p*-aminophenol was demonstrated by Brodie and Axelrod (29). Burns, *et al.* (131), report that a hydroxylated metabolite of butazolidin has a pronounced anti-inflammatory activity as well as being more toxic than the parent drug. A sulfoxide metabolite of the 4-phenylthioethyl derivative of butazolidin has a uricosuric effect much greater than the unchanged drug (132).

Axelrod (133) comments on the formation of active metabolites from endogenous substrates as well as from foreign compounds.

Of related interest is the development of medicinal compounds containing structural groups known to undergo enzymatic attack with release of the active species (drug latentiation) as discussed by Harper (134). A fuller understanding of the metabolism, storage, and transport of drugs and drug metabolites will make this area of medicinal chemistry a fruitful field.

SUMMARY

Studies cited in this review indicate a rapid development of knowledge concerning the biological fate of drugs and other foreign organic chemicals since 1954, coincident with the discovery of systems present in mammalian liver microsomes which catalyze a wide variety of drug metabolic pathways. It is now possible, using this *in vitro* system, to carry out more refined and precise studies of drug metabolism than was the case with organ perfusion techniques and isolation of metabolites from excreta. This subcellular system duplicates exactly the metabolites formed and excreted by the intact animal, so that no question of specificity is involved. The contributions of Brodie, Axelrod, Udenfriend, and their co-workers of the National Institutes of Health are highly significant in the development of current knowledge on drug metabolism.

While recognizing that drugs can be converted *in vivo* to metabolic products exhibiting pharmacological activity, many questions remain to be answered relative to the quantitative importance of such conversions. Modification of drug response through administration of compounds known to stimulate or inhibit the liver microsomal system is an interesting area of study, and probably of future clinical significance. A thorough understanding of the factors operative in the normal animal which control the synthesis and function of the microsomal enzymes must be established in order to open another avenue of effort in modifying drug response. Isolation and characterization of the enzymes involved in drug metabolism is of paramount importance. Further data on the apparent discrimination of the liver microsomal enzymes against endogenous substrates are required to establish this point firmly. Such information will be forthcoming only if studies are carried out with highly purified enzymes, which has not been the case to date.

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Study of Water Vapor Sorption by Pharmaceutical Powders

By W. A. STRICKLAND, Jr.

Water vapor sorption by pharmaceutical powders was studied by methods designed to yield data suitable for use in the preparation and stability testing of pharmaceuticals such as capsules, tablets, etc. The study yielded sorption-desorption isotherms near room temperature that permit the estimation of water sorption behavior of these powders in normal atmospheric conditions.

POWDERS may sorb water vapor from the surrounding atmosphere by physical and/or chemical bonding. The tenacity with which water is held by such powders as starch and certain metallic oxides is well known. The affinity of powders for water may be a factor of importance when considering the fabrication and stability of pharmaceutical preparations such as capsules, tablets, powders, and granules.

The literature reveals an abundance of work concerning sorption and diffusion of water and water vapor in such fields as textiles, foods, ceramics, and fuels, most of which has little application to the subject at hand. Leeson and Mattocks (1) recently studied the decomposition of aspirin at various water vapor levels. Babbitt (2) used a quartz spring sorption balance to study the sorption of water vapor by wheat. Dacey, *et al.* (3), used a similar apparatus to study the adsorption of water by saran charcoal. Hoover, *et al.* (4), applied polarization theory to sorption of water vapor by high polymers, principally protein. The surface area of porous solids such as soils, gels, and charcoals were determined from their water adsorption isotherms by Puri and Sharma (5).

The purpose of this work was to study the sorption and desorption of water vapor by representative pharmaceutical powders. This report describes methods used in the study and gives data obtained.

EXPERIMENTAL

Three distinct approaches were employed in this study, each will be discussed separately.

Screening

All powders were initially tested by placing accurately weighed, untreated samples of about 5 Gm., suitably spread in aluminum foil containers, into humidity chambers for a period of approximately 1 week. At the end of the equilibrium period, the samples were weighed a second time and loss or gain in weight noted. The humidity chambers, which consisted of large Pyrex glass desiccators, were kept in a room maintained at 21 ± 2°. The desired relative humidities were obtained by placing appropriate concentrations of sulfuric acid-water solutions (6) or saturated solutions of appropriate salts (7, 8) in the bottom of the desiccators. Corn starch was used as a reference in screening tests because it exhibited a predictable gain or loss of weight at various relative humidities.

Discussion of Results of the Screening Procedure.—Table I gives typical qualitative results of the screening procedure with powders that contain appreciable water or possess marked tendency to sorb water vapor. Powders showing such results were deemed suitable for further study. The amount of water held by the powder at 45% relative humidity was considered as a normal water content.

TABLE I.—RESULTS OF SCREENING TESTS SHOWING WATER VAPOR SORPTION OR DESORPTION ON UNTREATED POWDER SAMPLES

Compound	Gain or Loss of Weight, mg./Gm. Starting Weight, in Humidity Chambers				
	0% RH[a]	20% RH	45% RH	70% RH	90% RH
Casein	−50	−18	0	43	95
Magnesium trisilicate	−82	−20	3	17	30
Thiamine hydrochloride	−40	−6	2	4	30
Magnesium carbonate	−15	−2	0	8	22
p-Aminobenzoic acid	0	0	0	0	33
Methylene blue	−40	−2	0	113	135
Polyglycol E4000 (Dow)	−19	−18	−11	5	125
Polyglycol E20,000 (Dow)	−11	−10	−7	2	44
Sodium diphenylhydantoinate	−10	−2	4	239	291
Corn starch	−99	−24	2	40	88
Sodium phenobarbital	−16	..	6	..	91
Methocel 4000 cps. 90 Hg	−59	−20	10	69	141
Procaine base	0	0	0	0	154

[a] RH, relative humidity.

Received May 8, 1961, from the University of Arkansas, School of Pharmacy, Little Rock.
Accepted for publication June 30, 1961.
The author appreciates the suggestions and assistance of Drs. L. M. Wheeler and L. M. Lueck, Parke, Davis and Co.

If a powder neither gained nor lost weight it was presumed to be essentially free from water vapor sorption characteristics and was not further studied. Table II lists several powders that neither gained nor lost weight in these humidity chambers.

TABLE II.—POWDER SAMPLES THAT NEITHER GAINED NOR LOST WEIGHT (LESS THAN ±0.5%) DURING SCREENING TESTS IN HUMIDITY CHAMBERS

Sulfathiazole	Phenothiazine
Phenobarbital	Hydroquinone
Benzocaine	Diphenylhydantoin
Tetracaine base	Chloramphenicol
Talc	Codeine alkaloid
Quinine alkaloid	Holocaine hydrochloride
Cincophen	Prochlorperazine maleate
Niacin	Procaine hydrochloride
Niacinamide	Aminopyrine
dl-Amphetamine sulfate	Aminophylline

Determination of Water Vapor Sorption Isotherms in Humidity Chambers

Accurately weighed samples, approximately 2 Gm. each, of desiccated powders in tared glass weighing bottles of such diameter that the loosely spread powder was less than 0.5 cm. deep, were placed in a humidity chamber which consisted of a large Pyrex glass desiccator, and dried at 110° under reduced pressure for 4 hours, when melting points permitted. The powder samples, some of which were recrystallized, were seived through 100-mesh screens. After the final weighing, conducted in such manner as to avoid sorption of atmospheric water vapor, concentrated sulfuric acid was introduced into the lower portion of the chamber and the top sealed in place. Normal atmospheric pressure and content existed in the humidity chamber at all times during the determination. The assembly was placed in a constant temperature water bath maintained at 25 ± 0.2° and allowed to equilibrate for about 1 week. At the end of this time the powders were weighed swiftly on a projection balance with due caution to avoid sorption of atmospheric water vapor. Subsequently, solutions of sulfuric acid and water were introduced into the chamber bottom to give increases in relative humidity of 10–20% each week. An aliquot portion of this solution was titrated with standard base at each weighing time to determine precisely the equilibrium concentration. The relative humidity corresponding to concentration of sulfuric acid was found by reference to the International Critical Table (6).

The desorption isotherm was obtained by reducing the relative humidity in the chamber by weekly increments similar to those used in the sorption study.

Discussion of the Sorption and Desorption Isotherms Obtained in Humidity Chambers.—The shape of the isotherms obtained in this study (Figs. 1–4) were reproducible; however, precise reproduction of points on the curves was not usually achieved. Perhaps surfaces of the particles were altered during the several weeks of each study, for a second determination with a given sample did not yield precisely the same result as the first. Close agreements were obtained with successive samples of the same powder.

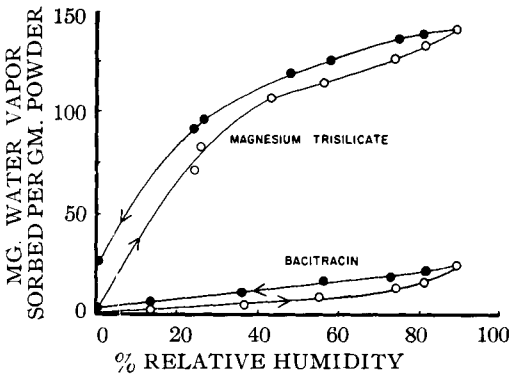


Fig. 1.—Sorption and desorption of water vapor in relative humidity chambers (25°) on 100-mesh powders. ○, Sorption; ●, desorption.

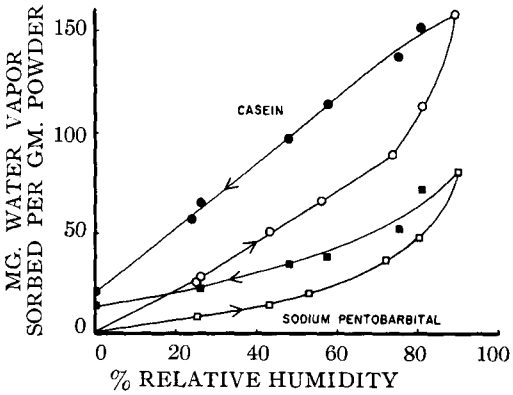


Fig. 2.—Sorption and desorption isotherms (25°) obtained in humidity chambers using 100-mesh powders. The closed points indicate desorption portion of curves.

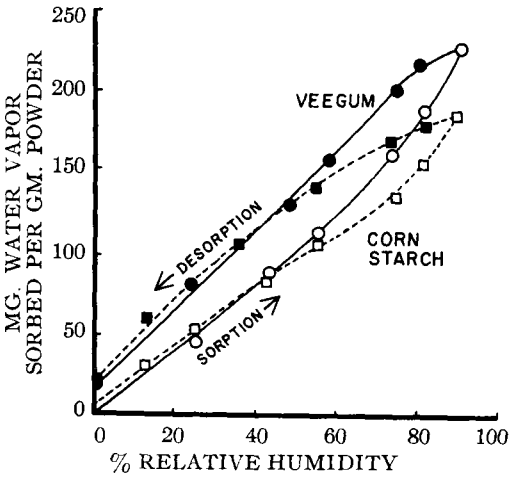


Fig. 3.—The sorption and desorption of water vapor observed in humidity chambers (25°). The Veegum (R. T. Vanderbilt Co., colloidal magnesium aluminum silicate) samples, used without sieving, consisted of particle sizes ranging from small flakes to fine powder.

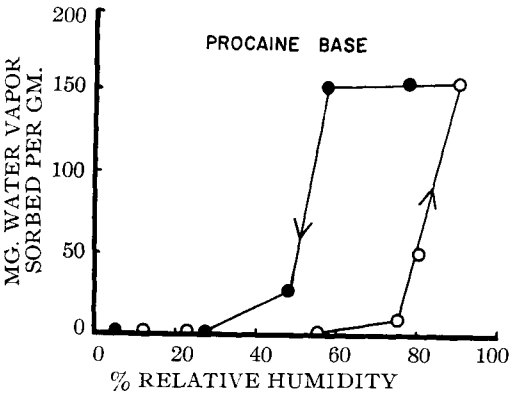


Fig. 4.—The sorption-desorption isotherm of procaine base powder, 100 mesh, in humidity chambers (25°) was influenced by formation of a dihydrate. This behavior was elucidated by further study in the quartz spring sorption apparatus. O, Sorption; ●, desorption.

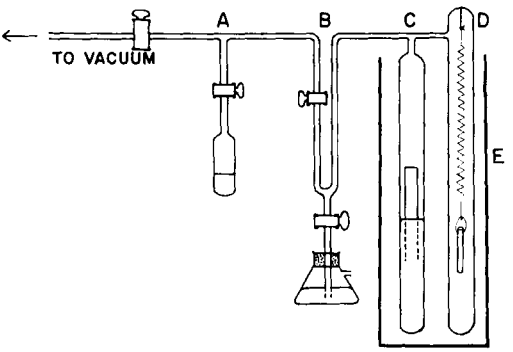


Fig. 5.—Diagram of the vapor sorption apparatus. A, Water vapor reservoir; B, mercury trap; C, Dubrovin gauge; D, sample chamber; E, water bath.

The pronounced hysteresis loop of the desorption curve found in many of these isotherms may be due in large measure to failure to attain equilibrium on the sorption curve and/or on the desorption curve. Hysteresis has been encountered in other systems and can be accounted for in different ways aside from lack of equilibrium, such as the "ink bottle" theory, but the hysteresis shown here was reproducible and it existed in the same magnitude even though extended periods were allowed for equilibration.

Determination of Water Vapor Sorption Isotherm with Quartz Spring Sorption Apparatus

The isotherm for procaine base (Fig. 4) was substantially different than that for most powders. Procaine base is known to form a dihydrate when recrystallized from ethanol-water solutions (9) and has been described as a hygroscopic powder (10); however, literature search did not reveal studies on the nature of this water vapor sorption. Consequently, an apparatus was constructed (Fig. 5) which permitted study of the mode of water vapor sorption and was capable of yielding data pertaining to the energy of hydration. The essential difference in the data obtained in this apparatus and that previously described was that this hydration takes place in the presence of water vapor alone and was not subject to the ambiguities introduced by the presence of atmospheric gases.

Equipment.—The apparatus (Fig. 5) consisted of two large connected tubes, one of which contained a quartz spiral spring from which the sample was suspended. The quantity of water sorbed by the powder was obtained gravimetrically by measuring the extension of the quartz spring, which had the following characteristics: unloaded length approximately 6 cm., extension of 1 cm. per 51 mg. added weight, and a maximum load of 2 Gm. The second tube contained a Dubrovin gauge (11) having a multiplication factor of 4.91. Both the extension of the quartz spring and the position of the Dubrovin tube were determined with a cathetometer which could be read to ±0.01 cm. The water bath, in which the assembly was immersed was maintained at 23.6 ± 0.1° by a temperature regulator-stirrer.

Procedure.—Recrystallized samples of procaine base were sieved through 100-mesh screen and dried under vacuum. An accurately weighed sample of approximately 1 Gm. was suspended, in a cylindrical waxed-paper container, from the lower hook of the quartz spring. After the sample and spring were sealed in place, the apparatus was evacuated at less than 0.1 cm. mercury pressure for approximately 3 hours, after which time the system was isolated by closing the stopcock and raising the mercury seal (Fig. 5). After initial readings of pressure and spring extension with the cathetometer, water vapor was introduced into the assembly from the water reservoir. Sorption appeared to be complete in about 8 hours, however, 24 hours was the usual time allowed for equilibration, after which pressure and spring extension were determined. Increments of 10 to 20 mg. of water vapor were admitted daily to the sample chamber. When the water vapor rose to about 2.10 cm. mercury corresponding to $p/p_0 = 0.96$ (p_0 for water at 23.6° is 2.185 cm. Hg) (12) the determination of the sorption isotherm was considered complete. The desorption isotherm was subsequently determined by removing daily increments of water vapor with the vacuum pump until the water vapor pressure was reduced to about 0.1 cm. of mercury.

Discussion of Results.—The sorption and desorption isotherms obtained in the quartz spring sorption apparatus were essentially identical and did not exhibit the hysteresis observed in results from humidity chambers. Since desorption data were somewhat more precise than that obtained on initial sorption, it was utilized in this report. The isotherm seen in Fig. 6 (data in Table III) indicated the formation of a dihydrate without evidence of a monohydrate, at an equilibrium water vapor pressure of 1.26 cm. Hg ($p/p_0 = 0.57$).

Equilibrium pressures obtained at other temperatures, shown in Table IV and plotted in Fig. 7 yielded a net ΔH (thermodynamic enthalpy of the reaction) of -29.6 ± 3 Kcal. per mole of procaine for the following reaction

$$\text{Procaine}_{(\text{solid})} + 2H_2O_{(\text{gas})} \rightarrow \text{Procaine}\cdot 2H_2O_{(\text{solid})}$$

The order of the reaction between water vapor and procaine base powder was investigated by

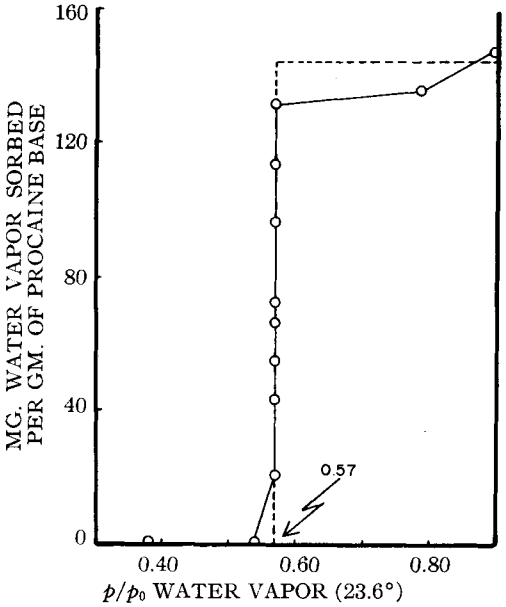


Fig. 6.—This isotherm shows the hydration of procaine base as it occurs in the presence of water vapor alone. The departure from theory at the upper portion of the isotherm may be due to failure to achieve equilibrium occasioned by the decreasing amount of unhydrated procaine base and the accompanying increase in difficulty of water vapor diffusion through the crystals as hydration nears completion. ---, Theory; ——, actual.

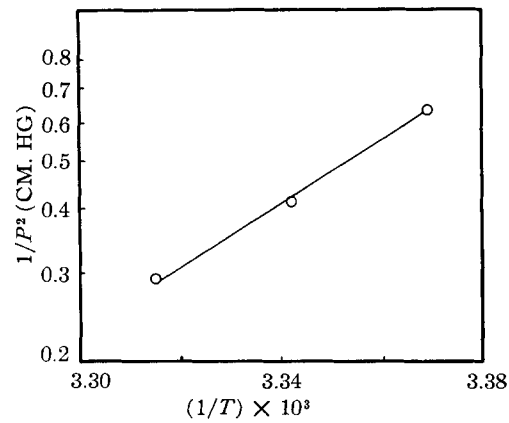


Fig. 7.—A Clausius-Clapeyron plot of temperature *vs.* equilibrium pressure between water vapor, procaine base, and procaine base dihydrate to obtain the energy evolved in this hydration (see Table IV).

studying the rate of hydration. This was accomplished by measuring the weight gain of the sample as a function of time while maintaining a relatively constant water vapor pressure of about 2.0 cm. Hg (corresponding to a p/p_0 of 0.90 to 0.95). The result, seen in Fig. 8, indicated a zero-order reaction. Perhaps the rate limiting factor was the diffusion of water molecules from the surface to the interior of crystals.

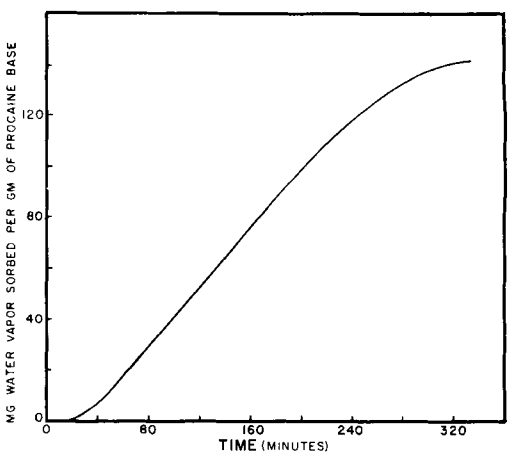


Fig. 8.—The rate of hydration of a sample of procaine base, 100-mesh powder, in water vapor (p/p_0 of 0.90-0.95) at 23.6°. The straight line portion of the curve indicated a zero-order reaction.

TABLE III.—EQUILIBRIUM PRESSURES OF WATER VAPOR WITH PROCAINE BASE OBTAINED IN THE QUARTZ SPRING SORPTION APPARATUS

Mg. of Water Sorbed per Gm. of Procaine Base	Water Vapor Pressure at Equilibrium, cm. Hg	p/p_0 at 23.6° (p_0 = 2.185 cm. Hg)
147	2.04	0.93
136	1.71	0.78
131	1.25	0.57
114	1.24	0.57
96	1.25	0.57
72	1.26	0.58
66	1.26	0.58
55	1.26	0.58
43	1.23	0.57
21	1.24	0.57
0	1.15	0.53
0	0.80	0.37
0	0.48	0.22

TABLE IV.—EQUILIBRIUM PRESSURES OF PROCAINE DIHYDRATE AND WATER VAPOR AT DIFFERENT TEMPERATURES

Temperature, °C.	$1/T \times 10^3$	Pressure, cm. Hg	$1/p^2$
23.6	3.369	1.26	0.630
26.0	3.342	1.56	0.411
28.5	3.315	1.85	0.292

CONCLUSIONS

The hygroscopic nature of several medicinal powders has been studied. The methods employed were designed to yield data suitable for use in the fabrication and study of pharmaceutical systems such as tablets, capsules, granules, and powders. The study of hygroscopic powders in relative humidity chambers yielded isotherms that exhibited hysteresis, indicating that sorption and desorption of water vapor by common

pharmaceutical powders was not a freely reversible process. Procaine base was cited as an example of powder exhibiting an unusual sorption of water vapor which can be accounted for by formation of a crystalline dihydrate.

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Standardization of Fibrinolytic Preparations

Measurement of Proteolytic and Activator Activity of Streptokinase-Activated Human Plasminogen

By WERNER BAUMGARTEN, JOSEPH L. CIMINERA, and SUZANNE M. VAN PELT

Methods for the determination of proteolytic and activator activity of streptokinase-activated human plasminogen are presented. These assay procedures extend previously published methods by the incorporation of a suitable standard and improved experimental design. The experimental bias is minimized and, by test of validity, differences in preparations can be realized.

IN THE LAST few years fibrinolytic agents have been developed which can assist in the clinical dissolution of venous and arterial clots. Several of these agents have become commercially available and, consequently, standardization of these preparations has become of considerable interest.

A fibrinolytic preparation[1] has been made commercially available by our laboratory. This is a streptokinase-activated human plasminogen preparation. The activity of fibrinolysin has been determined by a fibrinolytic assay procedure (1) which measures the total activity of the preparation. It has been established, however, that fibrinolysin possesses two distinct properties which contribute to its enzymatic activity (2–4). Streptokinase reacts with a proactivator present in human plasminogen producing an activator. The resulting activator then converts the proteolytic precursor which is also present in the human plasminogen into the active enzyme.

In the fibrinolytic assay the total activity is measured; however, it is possible to determine the proteolytic and activator activity separately.

Several procedures (5, 7–9) have been described in the literature; however, they are lacking in statistical treatment and omit the use of an appropriate standard. In our laboratory a self-contained slope ratio assay for the caseinolytic and activator activity of fibrinolysin preparations has been developed. A self-contained assay includes a reference standard and provides statistical procedures for testing the validity of the assay and methods for estimating the relative potency and error of the assay.

Over a limited range of concentration, fibrinolysin preparations give a linear response and in the assay, concentrations are chosen which fall on this linear portion of the curve.

Measurement of the proteolytic activity is done by the direct caseinolytic assay in which the degree of hydrolysis of the casein substrate is determined. In the activator assay added plasminogen is first converted into the active proteolytic enzyme by the activator present in fibrinolysin. The amount of activator which is required for the enzymatic conversion of the

Received April 27, 1961, from Merck Institute for Therapeutic Research and Merck Sharp and Dohme Research Laboratories, West Point, Pa.
Accepted for publication July 6, 1961.
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proteolytic precursor to the active enzyme does not produce measurable casein hydrolysis if plasminogen has been omitted from the reaction mixture. Thus, the hydrolysis of the casein substrate by the activated enzyme is, for all practical purposes, a measure of the activator activity of the fibrinolysin preparation.

MATERIALS AND METHODS

Reagents.—*Preparation of Casein.*—Fifty grams of casein (Hammarsten)[2] is suspended in 1 L. of distilled water and stirred vigorously for 1 hour. The mixture is centrifuged for 10 minutes at 1500 r.p.m. and the supernatant discarded. The casein is resuspended in 1L. of water and the process repeated. The casein is now suspended in 800 ml. of 95% ethanol (3A) and the mixture filtered by suction. The casein is washed twice on the filter with 95% ethanol followed by three washings with absolute ether. Finally, the casein powder is air dried.

Phosphate-Saline Buffer.—Potassium dihydrogen phosphate, 13.6 Gm.; sodium chloride, 9.0 Gm.; *q.s.* to 1L. with distilled water; adjust pH to 7.4 with 5 *N* sodium hydroxide.

Trichloroacetic Acid (TCA), 5% and 10%.

Sodium Carbonate, 20%.

Folin-Ciocalteu Phenol Reagent, Fisher Scientific Co., King of Prussia, Pa.

Preparation of Casein Solution for Assay.—Five grams of dried casein is suspended in 100 ml. of phosphate-saline buffer, pH 7.4, and heated for 15 minutes in a boiling water bath. After cooling, the mixture is centrifuged for 10 minutes at 1500 r.p.m., the supernatant is decanted, and its pH adjusted to 7.4 with 5 *N* sodium hydroxide. The casein solution may be kept in the refrigerator for one week prior to use.

Fibrinolysin Standard.—A number of vacuum sealed vials of fibrinolysin were stored in the refrigerator and designated "standard fibrinolysin preparation."[3] This standard is employed in the caseinolytic as well as in the activator assay.

In the caseinolytic assay a vial of fibrinolysin is quantitatively restored to 10 ml. with distilled water, and aliquots of 0.2 and 0.4 ml. are employed at the two levels of the assay.

For the activator assay the vial of fibrinolysin is restored to a final volume of 5,000 ml. As in the caseinolytic assay, 0.2 and 0.4 ml. of this solution are employed for the two concentrations of the standard.

Assay Procedure.—*Proteolytic Assay.*—The fibrinolysin standard (50,000 fibrinolytic units per vial) is dissolved in distilled water and brought to 10 ml. in a volumetric flask. The standard 0.2 and 0.4 ml. quantities, are pipetted into $^5/_8 \times$ 5-in. test tubes and the volume is adjusted to 1 ml. with phosphate-saline buffer. Tubes containing 0.2 ml. are labeled S_L and tubes 0.4 ml. are labeled S_H. In order to minimize loss of enzymatic activity on standing the tubes are immediately immersed into an ice bath.

The concentration of the test preparation is adjusted by an initial dilution in order to give responses similar to those for the standard. This requires a prior knowledge of the approximate activity of the test preparation. Aliquots of the test solution with the low concentration at one-half that of the high are added to assay tubes labelled T_L and T_H and brought to a volume of 1 ml. with phosphate-saline buffer.

Tubes can be set up in replicate. If duplicate determinations are judged sufficient, a total of three test preparations can be assayed simultaneously against the standard.

While in the ice bath the assay tubes are arranged in the order schematized in Figs. 1, 2, or 3, corresponding to quadruplicate, triplicate, and duplicate tubes, respectively. Figure 3 represents a restricted random allocation for three test preparations and a standard, so that the same duplicate tubes do not appear in the same row or column. An incubated blank containing 1 ml. of phosphate-saline buffer is included in every caseinolytic assay and may be placed at random in the test tube rack.

At zero time, 4.0 ml. of the 5% casein solution is added to the first tube starting at the lower left-hand corner, the solution is mixed, and the tube is placed into the water bath at 37° and incubated for 60 minutes. Casein is added to the remaining tubes at 60-second intervals, the tubes are mixed and placed in the water bath according to the designated scheme. Casein is added to the tubes going from left to right and ending with the tube in the upper right-hand corner. All subsequent manipulations follow the same order as above. The blank is added last. After an incubation of 60 minutes, 2 ml. of the incubated casein mixture is withdrawn from each tube and pipetted into 2 ml. of 10% trichloroacetic acid. The suspension is mixed well, allowed to stand at room temperature for at least 15 minutes, and filtered through S & S

A. Caseinolytic Assay (Blank—at Random)

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	T_H	T_L	S_L	S_H
R_3	S_H	S_L	T_L	T_H
R_4	T_L	S_H	T_H	S_L

Fig. 1.—For quadruplicate determinations.

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	T_H	T_L	S_L	S_H
R_3	S_H	S_L	T_L	T_H

Fig. 2.—For triplicate determinations.

	P_1	P_2	P_3	P_4
R_1	U_H	V_L	T_H	S_L
R_2	S_H	T_H	V_L	V_H
R_3	V_H	U_L	S_H	T_L
R_4	S_L	T_L	U_H	U_L

Fig. 3.—For duplicate determinations and three test preparations.

[2] Nutritional Biochemicals Corp., Cleveland 28, Ohio.
[3] Thrombolysin, 50,000 units per vial (1).

No. 576 filter paper by gravity.[4] The following mixture is prepared: 10 ml. distilled water, 3.0 ml. 20% sodium carbonate, and 1.5 ml. 5% TCA. A 0.5-ml. quantity of the filtered digestion mixture is added, followed by 1.0 ml. of the phenol reagent, and the contents of the tubes are mixed thoroughly.

Twenty minutes after the addition of the phenol reagent, the absorbance of the solution is read on a Beckman spectrophotometer at the wavelength of 650 mμ in a 5-cm. absorption cell with a light path of 50 mm. The readings are made against the casein blank.

Activator Assay.—This differs from the proteolytic assay. Human plasminogen is added to each tube and is allowed to react with the fibrinolysin standard or test preparation.

To each assay tube is added 3 mg. (0.3 ml.) of human plasminogen (fraction III-3) (9) and a predetermined volume of phosphate-saline buffer calculated to give a total volume of 1 ml. after taking into account the volume of the activator solution. The tubes are arranged according to the scheme in Fig. 4, which represents the same type of restricted random allocation as Fig. 3 except that only two test preparations are being assayed. This represents the maximum number of tubes that can be used practicably for this type assay.

B. Activator Assay (Blank—at Random)

	P_1	P_2	P_3	P_4
R_1	S_L	T_H	S_H	T_L
R_2	U_L	U_H	S_L	T_H
R_3	T_L	S_H	U_L	U_H

Fig. 4.—For duplicate determinations and two test preparations.

If determinations are desired in triplicate, the scheme in Fig. 2 may be utilized.

At 90-second intervals precooled 0.2-ml. and 0.4-ml. aliquots of the standard and appropriate aliquots of the test solution with the low concentration at one-half that of the high are added to the appropriate assay tubes, followed immediately by 0.3 ml. of the casein solution, going from left to right, starting in the lower left-hand corner and ending with the tube in the upper right corner. All subsequent manipulations follow the same order as above. Each tube is immediately incubated for 18 minutes at 37°. At that time 3.7 ml. of casein solution is added, and after an additional incubation of 2 minutes, a 2-ml. aliquot is pipetted into 2 ml. of 10% trichloroacetic acid. A second aliquot is removed after an additional 60-minute incubation.

The suspension is mixed well, allowed to stand at room temperature for at least 15 minutes, and filtered. The filtrate is treated as described under proteolytic assay.

Statistical Evaluation of a 4-Point Slope Ratio Assay.—Preliminary trials showed that absorbance was linearly related to concentration of fibrinolysin (i.e., followed the Beer-Lambert law) at least up to an absorbance of 0.8. Variation among replicate tubes was small and constant up to an absorbance of about 0.4; above this the variation became much larger and was less constant. We decided, therefore, to limit the concentration of test materials to such that the absorbance would not exceed 0.5.

A uniformity trial was run in which a standard preparation was assayed against itself. The concentrations tested were S_L = 0.2 ml., S_H = 0.4 ml., T_L = 0.15 ml., and T_H = 0.3 ml. Four tubes of each concentration were tested. The order of placement of the tubes in the water bath was, according to Fig. 1, completely randomized in order to minimize possible positional bias. A protocol illustrating the calculations is shown in Table I. This represents a simplified approximate procedure and the formulas are valid only for the standardized conditions of this assay; i.e., two concentrations of each preparation with the low concentration at one-half that of the high, and four tubes at each concentration. Similar computations are used for triplicate and duplicate tubes, except that the formulas for F and P* are as shown in Table II.

Before proceeding with the estimation of relative potency, it is necessary to make a test for validity of the assay. This is done by comparing the absolute value of $|P|$ with 7W/3. If $|P| \geq 7W/3$, this would indicate that the two response lines do not have a common intercept, and the assay would be fundamentally invalid at $P \leq 0.01$. If $|P| \geq 5W/3$, this would show fundamental invalidity at $|P| \leq 0.05$; however, for most assays, a lack of validity at this level may, for practical reasons, either be ignored or the assay repeated.

A significant lack of common intercept would indicate either that the response(s) for one of the preparations is outside the linear range of response, or else that other factors such as inhibitors or potentiators are present which may modify the response of that preparation. If the apparent lack of common intercept is due to the former, the situation may be corrected by a better selection of concentrations.

The 95% confidence limits estimated from the intrinsic error of the assay may not necessarily reflect the "true" error of the assay. Weighing, dilution, and other technical errors may be larger than the intrinsic error. A more realistic estimate would require the combination of estimates from several replicate assays.

DISCUSSION

Kunitz (10) developed a method for the determination of trypsin based on its ability to digest a casein substrate. Remmert and Cohen (5) adopted this procedure and developed an assay for streptokinase activated human plasminogen. Succeeding authors introduced minor modifications.

The present assay procedure is patterned after that of Remmert and Cohen. It was found that results of identical samples submitted for assay to several independent laboratories varied considerably and it was thought advisable to standardize the assay procedures.

Arbitrarily, a standard fibrinolysin preparation was assigned a unit value and this preparation serves as a house standard in our laboratory. This fibrinolysin is prepared by streptokinase activation of human plasminogen. Earlier authors have ex-

[4] The suspension or filtrate may stand overnight in the refrigerator.

TABLE I.—PROTOCOL FOR 4-POINT SLOPE RATIO ASSAY

Preparation	Standard		Test	
Concentration	0.2 ml.	0.4 ml. = X_S	0.15 ml.	0.3 ml. = X_T
Absorbances	0.155	0.363	0.105	0.269
	0.149	0.378	0.103	0.260
	0.140	0.356	0.096	0.277
	0.146	0.366	0.109	0.273
Sum	$S_L = 0.590$	$S_H = 1.463$	$T_L = 0.413$	$T_H = 1.079$
Range	0.015	0.022	0.013	0.017

$X = X_S/X_T = 0.4/0.3$

W = sum of ranges $= 0.015 + 0.022 + 0.013 + 0.017 = 0.067$

$N = 3S_H - 6S_L + 7T_H - 4T_L = (3)(1.463) - (6)(0.590) + (7)(1.079) - (4)(0.413) = 6.750$

$D = 7S_H - 4S_L + 3T_H - 6T_L = (7)(1.463) - (4)(0.590) + (3)(1.079) - (6)(0.413) = 8.640$

$K = N/D = 6.750/8.640 = 0.78125 \quad K^2 = (0.78125)^2 = 0.61035$

$P = 2S_L - S_H - 2T_L + T_H = 2(0.590) - 1.463 - (2)(0.413) + 1.079 = -0.030$

$$F = \frac{5.5W}{D}\sqrt{1 - 1.64K + K^2} = \frac{5.5(0.067)}{8.640}\sqrt{1 - 1.64(0.78125) + 0.61035} = 0.02431$$

$P^*_{0.05} = 5W/3 = 5(0.067)/3 = 0.112 \quad P^*_{0.01} = 7W/3 = 7(0.067)/3 = 0.156$

test for validity of assay

since $|P| = 0.030$ is less than $7W/3 = 0.156$, assay is valid

estimation of relative potency

$R = XK = (0.4/0.3)(0.78125) = 1.04$

estimation of 95% confidence limits

$R_L, R_U = X(K \pm F)$

$= (0.4/0.3)(0.78125 \pm 0.02431)$

$= (0.4/0.3)(0.75694, 0.80556)$

$= 1.01, 1.07$

TABLE II.—FORMULAS FOR F AND P* FOR REPLICATE TUBES

Replicates	F	P* 0.05	P* 0.01
2	$\frac{8.75W}{D}\sqrt{1 - 1.64K + K^2}$	13W/5	22W/5
3	$\frac{6W}{D}\sqrt{1 - 1.64K + K^2}$	9W/5	8W/3
4	$\frac{5.5W}{D}\sqrt{1 - 1.64K + K^2}$	5W/3	7W/3

pressed their results in caseinolytic units. This unit was defined as the increase in absorbance at 280 mμ after 60 minutes of incubation at 37° and conversion of the absorbance to micrograms of acid-soluble tyrosine by reference to a standard curve. This procedure possesses definite disadvantages. Changes in experimental condition such as temperature of incubation, presence of inhibitors, and variations in casein substrate do not necessarily become apparent due to the lack of comparison to a standard response.

The new procedure evaluates fibrinolytic preparations by a self-contained statistical assay. The validity of the assay is evaluated and confidence limits are determined.

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Separation and Identification of Barbiturates and Some Related Compounds by Means of Gas-Liquid Chromatography

By E. BROCHMANN-HANSSEN and A. BAERHEIM SVENDSEN

Barbiturates and certain related hypnotics have been separated and identified by direct gas chromatography on a number of different stationary phases. Depending on the type and concentration of stationary phase, column temperatures ranging from about 140 to 200° were used. No evidence of decomposition of the substances was observed. The method is rapid and extremely sensitive.

RELATIVELY PURE single barbiturates can easily be identified by a number of methods, including the melting points of derivatives (1–4), infrared spectrophotometry (5–9), X-ray diffraction (10–15), and crystallography (16–20). When several barbiturates are present in a mixture, or when the material is contaminated with decomposition products and/or substances of biological origin, it is usually necessary to perform a separation prior to the identification process. Fractional extraction (21) and countercurrent distribution (22) have been used with some degree of success. Column partition chromatography (23) and adsorption chromatography (24) have given good results for specific problems of limited complexity. However, paper chromatographic procedures with a variety of solvent systems have proved the most satisfactory for more difficult separations (25–29). Yet, there are several combinations of barbiturates which have not been adequately resolved by this method. Furthermore, paper chromatography is somewhat time-consuming and fairly insensitive with respect to barbiturates.

In recent years, gas-liquid chromatography has proved to be an exceptionally valuable tool for qualitative and quantitative analysis of many types of compounds. Development of highly sensitive detectors and the use of columns containing low concentrations of the stationary phase have made the gas chromatographic method applicable to substances of very low volatility.

Attempts to identify barbiturates by gas chromatography have been reported by Janák (30, 31). He pyrolyzed the sodium salts and gas chromatographed the pyrolysis products. This paper describes a method for direct gas chromatographic separation and identification of barbituric acid derivatives and some related substances (32).

EXPERIMENTAL

A Barber-Colman model 15 gas chromatograph equipped with an argon ionization detector was used for the experimental work. The columns were glass U-tubes, 6 feet in length, and having an inner diameter of 3 mm. The solid support was Chromosorb W, 60–80 mesh, washed with concentrated

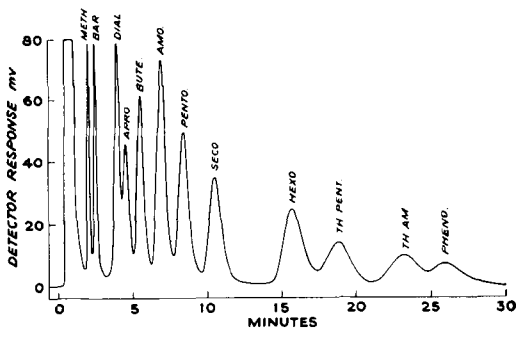


Fig. 1.—Gas chromatogram of 12 barbiturates on Apiezon L. The experimental conditions and the names of the barbiturates refer to Table I.

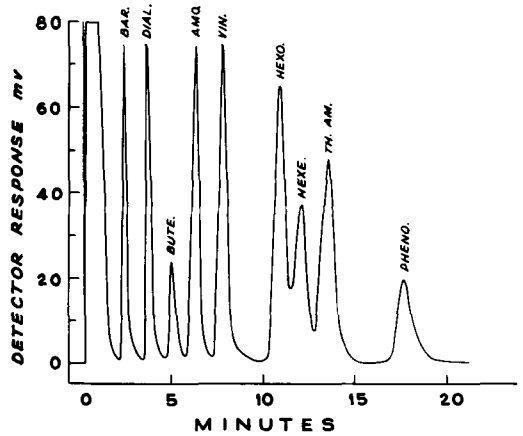


Fig. 2.—Gas chromatogram of nine barbiturates on SE-30. The experimental conditions and the names of the barbiturates refer to Table I.

Received July 25, 1961, from the University of California School of Pharmacy, Medical Center, San Francisco 22.
Accepted for publication September 18, 1961.
This work was supported by a research grant (M-3487) from the National Institutes of Health.

After this paper was submitted for publication, a paper entitled "Separation and Identification of Barbiturates by Gas Chromatography," by K. D. Parker and P. L. Kirk appeared in *Anal. Chem.*, 33, 1378(1961).

TABLE I.—RELATIVE RETENTION TIMES OF BARBITURATES AND RELATED COMPOUNDS ON COLUMNS WITH VARYING POLARITY

Compound	Apiezon L.	SE-30[a]	SE-52[b]	NGSE[c]	PEG 4000[d]	QF-1-0065[e]
Allylisopropylacetylurea	0.40	0.45	0.43	...	...	...
Amobarbital	0.82	0.92	0.90	0.92	0.92	0.96
Aprobarbital	0.55	0.56	0.56	0.76	0.85	0.63
Barbital	0.29	0.32	0.33	0.50	0.53	0.47
5(2-Br. allyl)-5-isopropyl. b.a.	1.77	1.91	1.92	2.62	4.30	1.80
Butethal	0.65	0.71	0.70	0.86	0.84	0.80
Cyclobarbital	3.42	3.03	3.02	2.72	4.32	2.50
Cyclopal	2.06	1.82	1.86	2.03	3.14	1.47
Diallylbarb. acid	0.46	0.52	0.52	0.78	0.97	0.57
Glutethimide	1.98	1.36	1.46	0.86	0.53	1.67
Hexethal	2.15	1.94	1.84	1.51	1.66	1.51
Hexobarbital	1.88	1.61	1.65	0.89	0.62	1.54
Itobarbital	0.68	0.71	0.70	0.89	0.91	0.74
Mephobarbital	2.30	1.91	1.97	1.25	1.04	1.67
Metharbital	0.24	0.23	0.24	0.21	0.11	0.34
Pentobarbital	1.00	1.00	1.00	1.00	1.00	1.00
Phenobarbital	3.10	2.88	2.95	4.14	8.78	2.50
Probarbital	0.44	0.42	0.44	0.63	0.65	0.57
Sec. Butyl Br. allyl. b.a.	3.39	2.98	3.02	3.41	5.54	2.41
Secobarbital	1.26	1.29	1.27	1.17	1.38	1.09
Thiamylal	2.82	2.05	2.05	1.63	1.41	1.09
Thiopental	2.21	1.59	1.60	1.32	1.08	1.01
Vinbarbital	0.99	1.17	1.16	1.13	1.45	1.12
Temperature, ° C.	150	137	144	200	170	165
Inlet pressure, p.s.i.	20	25	20	20	20	16
Outlet flow rate, ml./min.	37.5	68	47	58	40	25
Support, mesh	60–80	60–80	60–80	60–80	60–80	60–80
Liquid phase, w/w %	1.4	1.2	1.4	1.7	0.6	2.8
Pentobarbital time, min.	8.4	6.6	6.3	6.4	7.4	7.6

[a] Methyl silicone polymer, General Electric Co. [b] Phenyl silicone polymer, General Electric Co. [c] Neopentyl glycol sebacate. [d] Polyethylene glycol 4000. [e] Fluorosilicone fluid QF-1-0065, 10,000 cs., Dow Corning Corp.

hydrochloric acid and methanolic potassium hydroxide, and treated with hexamethyldisilazane to reduce adsorptive effects (33). The stationary phase was applied by means of a solution in toluene or acetone as described by Horning, *et al.* (34).

The barbiturates were introduced with a Hamilton microsyringe as 1.0 μl. of a 0.5 to 1.0% solution in acetone. Sodium salts of barbiturates were converted to free acids by means of dichloroacetic acid in acetone (35).

The results for 21 barbiturates and two related compounds, glutethimide and allylisopropylacetylurea in several stationary phases ranging from nonpolar to very polar materials are shown in Table I. Pentobarbital was used as standard for calculation of the relative retention times. The temperature and pressure were adjusted so as to produce nearly the same retention time for pentobarbital on all columns. This required a temperature of 200° for neopentyl glycol sebacate, while about 140° was sufficient for the silicone rubber materials. Most substances investigated gave sharp single component peaks consistent with absence of decomposition. In a few instances, one or two small additional peaks were observed due, presumably, to impurities in the samples available. The efficiency of the gas chromatographic method for separation of mixtures of barbiturates is illustrated in Figs. 1–4. Amobarbital and pentobarbital which are difficult to separate by paper chromatography, are separated nicely on an Apiezon L column (Fig. 1). Figure 4 shows a gas chromatogram obtained with nonlinear temperature programming from 150 to 225° on a polyester column. Temperature programming extends the range of application, shortens the time, and makes the component peaks sharper. Not all

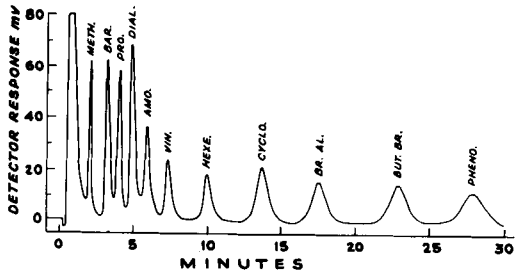


Fig. 3.—Gas chromatographic separation of 11 barbiturates on NGSE. The experimental conditions and the names of the barbiturates refer to Table I. CYCLO = cyclopal.

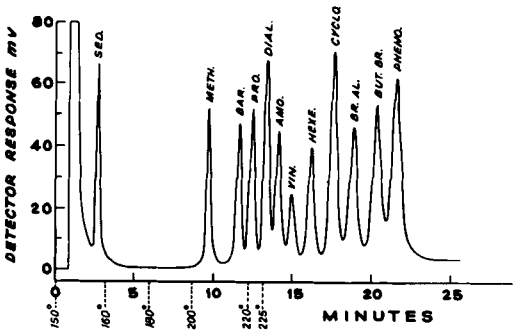


Fig. 4.—Gas chromatogram of 11 barbiturates and allylisopropylacetylurea (Sedormid) on NGSE obtained by nonlinear temperature programming.

substances examined could be separated on the same column. However, by using two columns, one with a nonpolar stationary phase such as Apiezon and one with a moderately polar polyester phase, a large number of possible combinations could easily be separated. The components were identified on the basis of their relative retention times (Table I). This method was used for identification of barbiturates in a number of pharmaceutical preparations.

DISCUSSION

The rate at which a solute travels through a gas chromatographic column depends on the vapor pressure of the pure compound and its activity coefficient in the stationary phase. With a nonpolar stationary phase where there is little or no interaction between solute and solvent, the separation in the column is largely a function of the vapor pressures of the pure substances. With an increase in the polarity of the stationary phase, various types of solute-solvent interaction take place, and the activity coefficient, expressing the lack of ideality of the solution, becomes an important component of the partition coefficient (36). For example, phenobarbital is eluted before cyclobarbital on an Apiezon column, as one might expect on the basis of their relative vapor pressures. On a polar column, such as a polyester or polyethylene glycol, the stationary phase causes more polarization of the phenyl ring than of the cyclohexenyl ring, and the elution sequence is reversed. A similar situation exists in the case of pentobarbital and vinbarbital which only differ by a double bond in one of the substituents in position 5.

Pentobarbital and secobarbital can easily be separated from their corresponding thio analogs on a nonpolar liquid (Fig. 1), but not on a polar liquid such as polyethylene glycol.

In Fig. 5 the relative retention times of a selection of barbiturates are plotted against the stationary phases arranged in the order of increasing polarity. The slopes of the curves indicate the degree of interaction between solute and solvent relative to that of pentobarbital. A positive slope would mean an increase in the activity coefficient over that of pentobarbital, and vice versa. As one would expect, the 1-substituted barbiturates, as well as the 2-thio barbiturates, exhibit negative slopes.

Dow Corning fluorosilicone fluid QF-1-0065 is a polar compound and has been used successfully for separation of steroids because of its great selectivity (37). It did not prove to be of any particular value for separation of barbiturates. It behaved as a very polar liquid in preventing the separation of pentobarbital and secobarbital from their corresponding thio analogs. On the other hand, it showed nonpolar behavior toward phenobarbital and cyclobarbital and similarly related compounds.

The gas chromatographic method gives promise of being an excellent tool, not only for analysis of barbiturates in pharmaceutical dosage forms, but also for fundamental studies of their rates and mechanisms of *in vitro* and *in vivo* transformations.

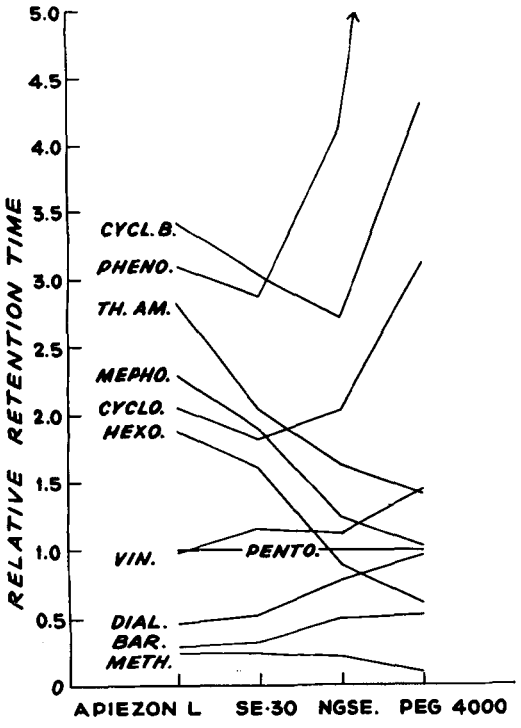


Fig. 5.—Relative retention times for 11 barbiturates plotted against stationary phases arranged in the order of increasing polarity. The experimental conditions and the names of the barbiturates refer to Table I.

SUMMARY

Twenty-three barbiturates and related compounds, including glutethimide and allylisopropylacetylurea have been gas chromatographed on columns containing stationary phases of varying polarity. By means of two columns, one with Apiezon L and one with a moderately polar polyester, most commonly used barbiturates can be separated. This method has been used for rapid identification of barbiturates in pharmaceutical preparations.

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Color Stability of Tablet Formulations V

Effect of Ultraviolet Absorbers on the Photostability of Colored Tablets

By L. LACHMAN, T. URBANYI, S. WEINSTEIN, J. COOPER, and C. J. SWARTZ

The photostabilizing effect of the ultraviolet absorber 2,4-dihydroxybenzophenone for tablets colored with three certified dyes was evaluated. Two sources of light were employed; one approximating the spectral energy distribution of sunlight and the other simulating ordinary room illumination. The apparent rates of fading for FD&C Yellow No. 5, FD&C Blue No. 1, and FD&C Red No. 3 were determined under each light source. The relationship existing between the protective properties of the ultraviolet absorber, the absorption characteristics of the dyes, and the spectral energy distribution of the artificial light sources is discussed.

THE STATUS of certified colorants continues to be closely scrutinized by the Food and Drug Administration. With the delisting of FD&C Red No. 1, there remain 11 certified dyes available for use in foods, drugs, and cosmetics. Temporary authorization for the continued use of FD&C and D&C colorants has been given, subject to the possibility that such use may be terminated or restricted. In view of the pending restrictions inherent with the use of colorants, maximum utilization of the certified dyes has become essential. This has stimulated interest in the pharmaceutical industry for developing methods of improving the stability of the limited number of available certified dyes.

One approach for improving the photostability of dyestuffs has been the use of ultraviolet absorbing chemicals. Chemical compounds such as 3,5-dinitrobenzoic acid (1), benzoyl resorcinols (2), alkylated 2-hydroxyphenylbenzotriazole, and derivatives of 2-hydroxybenzophenones (3) have been employed effectively as photostabilizing agents in industries such as textiles, plastics, paints, and cosmetics. These compounds are selected because they possess high absorptivity and good stability in the 300–400 mμ ultraviolet region without absorbing in the 400–800 mμ visible region. Since many FD&C colorants absorb radiation in the near ultraviolet and visible region of the spectrum, the use of an ultraviolet absorber in conjunction with these colorants should result in some stabilizing influence. This effect, however, would be governed by the spectral energy distribution of the light source used and the absorption characteristics of the colorants.

This study was initiated to determine the influence of an ultraviolet absorber, namely 2,4-dihydroxybenzophenone, on the photostability of tablets colored with three FD&C dyes representing different chemical classes. The colorants were selected so that they would exhibit absorption maxima throughout the visible spectrum (430, 540, and 640 mμ). The photostability of

Received April 28, 1961, from the Research Department, Ciba Pharmaceutical Products, Inc., Summit, N. J.
Accepted for publication July 24, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.

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these colorants in tablets with and without an ultraviolet absorber was studied upon exposure to two light sources, one approximating the spectral energy distribution of sunlight and the other ordinary artificial room illumination under normal and exaggerated light intensity.

EXPERIMENTAL

Materials.—FD&C Blue No. 1 (triphenylmethane dye), FD&C Red No. 3 (xanthene dye), and FD&C Yellow No. 5 (pyrazolone dye), H. Kohnstamm Co. 2,4-Dihydroxybenzophenone, Antara Chemical Co.

Equipment.—Gossen Tri Lux foot candle light meter, model C. Stokes model F tablet press. Beckman spectrophotometer model DU with reflectance attachment (5). Light stability cabinets exhibiting normal and exaggerated illumination. Light cabinet utilizing a General Electric type R.S. sunlamp (similar to the cabinet employed for normal illumination studies). In a previous report (4) a detailed description and discussion were presented on the light sources employed to simulate normal and exaggerated room illumination. However, a description of the light source used to simulate sunlight has not hitherto been presented. The lamp used is a self-contained unit with a 100-watt mercury discharge element and a 175-watt tungsten-filament resistance ballast incorporated into an ultraviolet transmitting reflector type bulb. The sunlamp emits energy in the ultraviolet and visible spectrum as shown in Fig. 1. It is interesting to note that its visible energy spectrum encompasses approximately the same wavelength range as does the cool white fluorescent lamp used in the normal illumination cabinet. The peak energy output for the sunlamp within this spectral range is greater than that emitted by the fluorescent lamp. The sunlamp is made with special glass which absorbs the shorter ultraviolet rays up to 280 mμ, thus simulating atmospheric absorption of the shorter violet rays emitted by the sun.

Preparation of Tablets.—Tablets containing water-soluble dyes with and without ultraviolet absorbers were prepared according to the following formulas

	With Ultraviolet Absorber, %	Without Ultraviolet Absorber, %
Calcium sulfate dihydrate	94.49	96.99
Tragacanth U.S.P.	2.00	2.00
2,4-Dihydroxybenzophenone	2.50	...
Magnesium stearate U.S.P.	1.00	1.00
Dye	0.01	0.01
Ethyl alcohol 50%	*q.s.*	*q.s.*

Granulations were prepared in accordance with commonly employed tabletting techniques. The colorants and the ultraviolet absorber were dissolved in 50% alcohol which was then used as the granulating solution. The granulations were dried to a moisture content below 0.5% and compressed into tablets using $^{8}/_{32}$ inch flat faced punches, uppers bisected, on a Stokes model F tablet press. The tablets were compressed to a weight of 100 mg. with a thickness of 1.5 mm. and a hardness of 8 Kg./in.^{2}.

Storage of Samples.—Samples of each of the colored tablets, with and without ultraviolet absorber, were placed into glass Petri dishes and then stored in light cabinets simulating normal and exaggerated room illumination. Samples were also stored in a light cabinet in which a General Electric type R.S. sunlamp emitted light with a spectral energy distribution simulating sunlight. The heat radiated by the sunlamp was dissipated by an integrated blower system which maintained the temperature in the cabinet at 40°. The intensity of the light incident on the tablets in the respective cabinets was kept at a constant level through measurement with a Gossen Tri Lux meter at regular time intervals. The light intensities on the tablets in the normal and exaggerated light cabinets were 45 and 550 foot candles, respectively, with a spectral energy range from 350 to 700 mμ (Fig. 1). The intensity of light incident on the tablets in the cabinet using the sunlamp was 700 foot candles with a spectral range of 280 to 700 mμ (Fig. 1). Tablet samples were removed from the cabinets at designated time intervals for reflectance measurements.

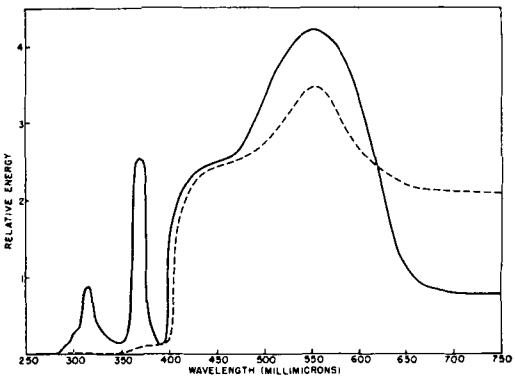


Fig. 1.—Spectral energy distribution of R.S. sunlamp and standard cool white fluorescent lamp. ——, R.S. sunlamp; – – –, cool white fluorescent lamp.

Measurement of Tablet Reflectance.—The procedure and equipment employed to follow changes in reflectance at the surface of the tablet were described in previous reports (5, 6). Reflectance measurements were taken at 430 mμ for FD&C Yellow No. 5, 540 mμ for FD&C Red No. 3, and 640 mμ for FD&C Blue No. 1.

RESULTS AND DISCUSSION

The protection provided by 2,4-dihydroxybenzophenone on the light-induced fading of tablets colored with FD&C Blue No. 1, FD&C Red No. 3, and FD&C Yellow No. 5 was evaluated after exposure to three lighting conditions. This ultraviolet absorber was chosen because it did not impart any significant color to the tablets, exhibited good ethanol solubility, was nontoxic, and had high absorptivity throughout the ultraviolet region and into the violet-blue region of the visible spectrum. Representative curves showing the transmission characteristics of this ultraviolet absorbing chemical at 0.025% and 0.25% concentrations in methanol are presented in Fig. 2.

The plots in Figs. 3–8 show the influence of sunlamp illumination and normal and exaggerated room

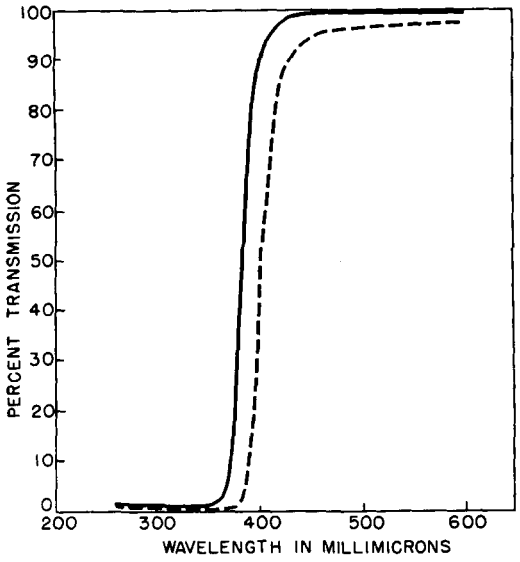


Fig. 2.—Absorption characteristics of ultraviolet absorber 2,4-dihydroxybenzophenone. ——, 0.025%, 2,4-dihydroxybenzophenone 400 in methanol; – – –, 0.25%, 2,4-dihydroxybenzophenone 400 in methanol.

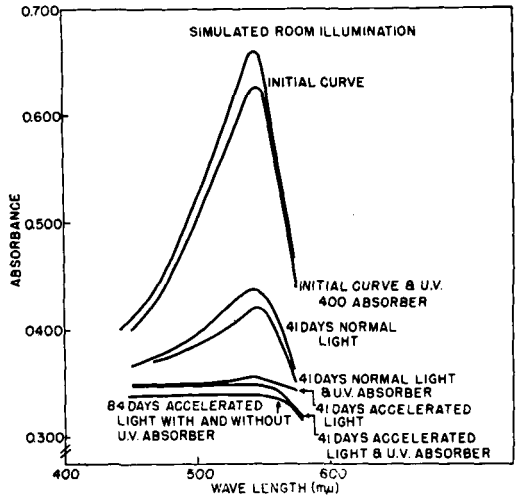


Fig. 3.—Visible absorption spectra of FD&C Red No. 3 after intervals of storage under normal and exaggerated illumination.

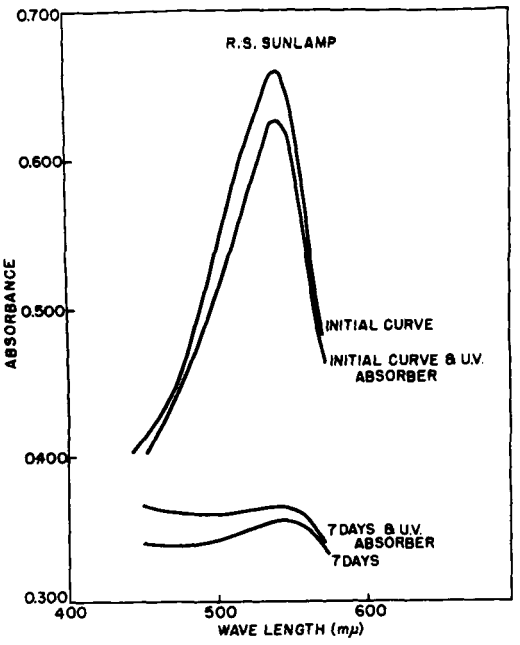


Fig. 4.—Visible absorption spectra of FD&C Red No. 3 after intervals of storage under simulated sunlight.

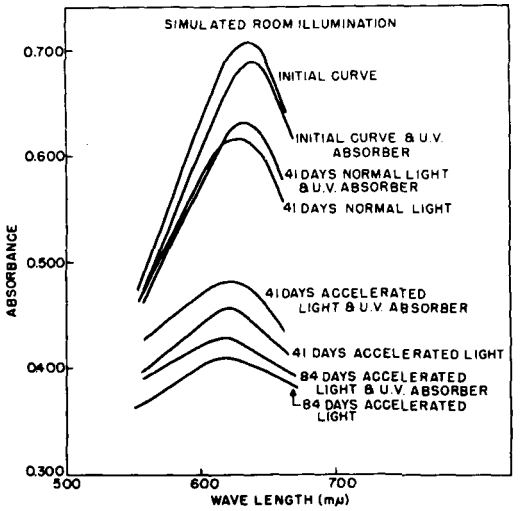


Fig. 5.—Visible absorption spectra of FD&C Blue No. 1 after intervals of storage under normal and exaggerated illumination.

lighting on the spectral absorbance at the surface of the colored tablets. It is apparent from Figs. 3 and 4 that the ultraviolet absorber does not significantly protect tablets colored with FD&C Red No. 3 when these are exposed to the three light sources. It is evident, however, that the tablets exposed to the radiations emitted from the sunlamp undergo more rapid fading than those exposed to exaggerated room lighting. This was also found to be true for the tablets colored with FD&C Blue No. 1 and FD&C Yellow No. 5.

The tablets colored with FD&C Blue No. 1 containing an ultraviolet absorber, were significantly protected from fading when exposed to each light source. The greatest protection occurred with regard to radiations from the sunlamp, as shown in Figs. 5 and 6. In studying the curves in Fig. 5, it is difficult to explain the protective action of the ultraviolet absorber for the FD&C Blue No. 1 dye when considering its ineffectiveness with FD&C Red No. 3. In fact, the absorption maximum for FD&C Blue No. 1 is at 640 mμ which is about 100 mμ further in the visible spectrum than FD&C Red No. 3. However, by studying the complete absorption curve for FD&C Blue No. 1 as shown in Fig. 6, it becomes readily apparent that the blue color has two absorp-

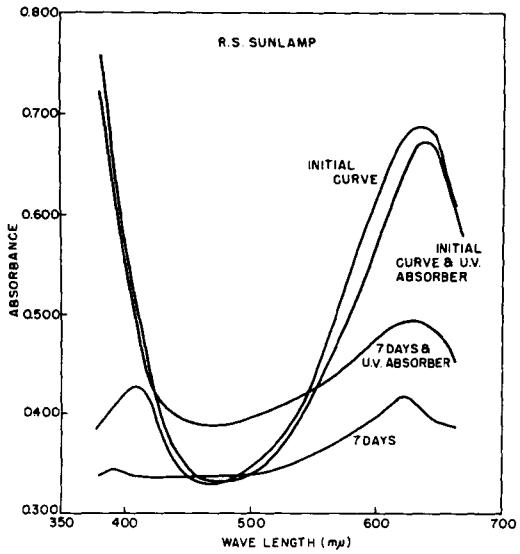


Fig. 6.—Visible absorption spectra of FD&C Blue No. 1 after intervals of storage under simulated sunlight.

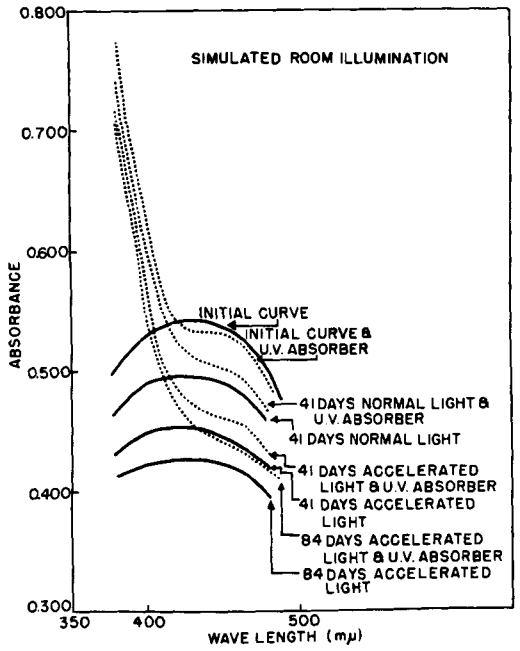


Fig. 7.—Visible absorption spectra of FD&C Yellow No. 5 after intervals of storage under normal and exaggerated illumination.

tion maxima, one at 640 mμ and another approaching the near ultraviolet 410 mμ. The absorption maximum at 640 mμ is much stronger than the one at 410 mμ. However, because of the strong absorbing tendencies of the ultraviolet absorber in the vicinity of 410 mμ peak of blue color, it would tend to protect this portion of the absorption curve of the dye. In turn, this would lessen the extent of fading at the absorption maximum of 640 mμ. This effect is clearly shown in the absorption curves obtained for

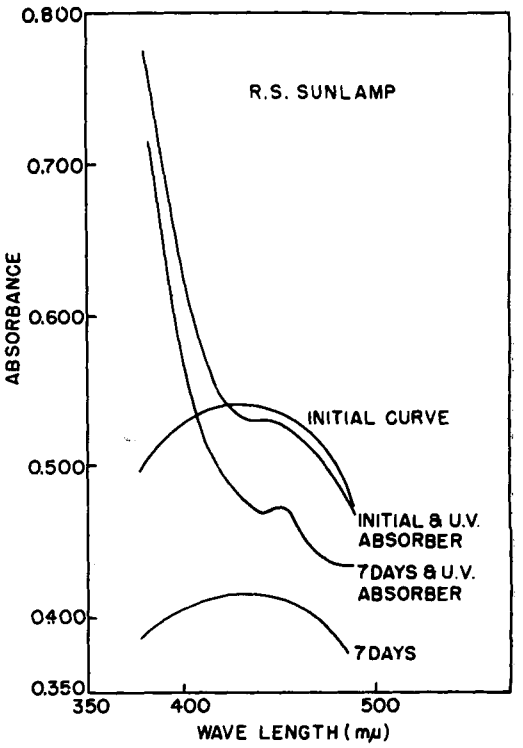


Fig. 8.—Visible absorption spectra of FD&C Yellow No. 5 after intervals of storage under simulated sunlight.

the tablets exposed to the radiations from the sunlamp (Fig. 6).

Of particular interest are the absorption curves obtained from the tablets colored with FD&C Yellow No. 5. It is evident from the curves in Figs. 7 and 8 that the absorption characteristics of the yellow color in tablets containing the ultraviolet absorber are drastically altered. This change can be ascribed to the strong absorbing properties of the 2,4-dihydroxybenzophenone in the vicinity of the absorption maximum of the yellow dye, which causes the overall spectral curve to take on the characteristics of the ultraviolet absorber.

The ultraviolet absorbing chemical exhibited its strongest stabilizing effect for the tablets colored with FD&C Yellow No. 5. This can be attributed to the close proximity of the absorption maximum of the color to that portion of the spectrum where the ultraviolet absorber exerts its strongest protective properties.

Representative plots showing the effect of the ultraviolet absorber on the rate of fading of tablets colored with FD&C Blue No. 1 and exposed to R.S. sunlamp and normal and exaggerated room illumination are presented in Figs. 9 and 10. From the linear regression of absorbance with time, it is apparent that the fading is proceeding according to apparent first-order kinetics. The plots show more than one slope and, in each instance, the initial rate of fading was greater than that obtained from the subsequent sections of the curve. Similar plots were obtained for FD&C Yellow No. 5 and FD&C Red No. 3. These results are similar to that found in our previous studies (6–8) and to those found by Baxter, *et al.*

TABLE I.—INFLUENCE OF AN ULTRAVIOLET ABSORBER ON THE RATE OF FADING OF COLORED COMPRESSED TABLETS STORED UNDER DIFFERENT LIGHT SOURCES

Color	Ordinary Room Illumination, Days × 10^3		Exaggerated Room Illumination, Days × 10^3		R. S. Sunlamp Illumination, Days × 10^3	
	With U.V. Absorber	Without U.V. Absorber	With U.V. Absorber	Without U.V. Absorber	With U.V. Absorber	Without U.V. Absorber
FD&C Blue No. 1	3.0	4.3	14.3	16.9	64.9	252
FD&C Red No. 3	34.5	36.1	166	152	565	576
FD&C Yellow No. 5	1.8	2.3	2.4	5.8	9.4	125

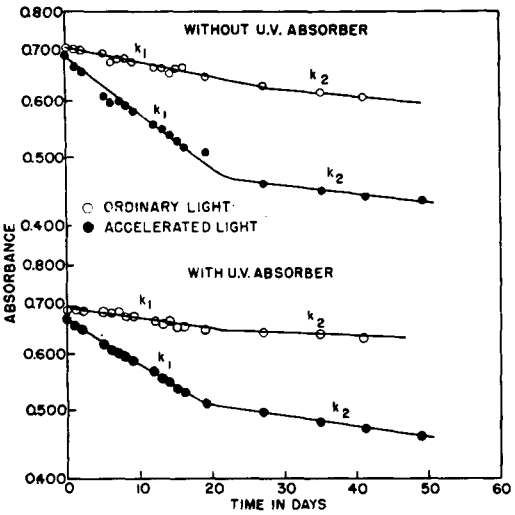


Fig. 9.—Influence of normal and exaggerated room illumination on the fading of the surface of tablets colored with FD&C Blue No. 1.

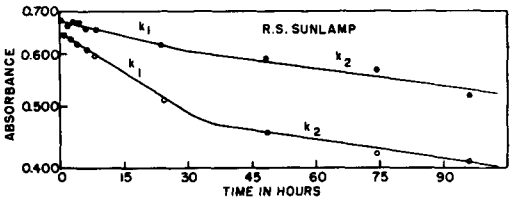


Fig. 10.—Influence of simulated sunlight on the fading of the surface of tablets colored with FD&C Blue No. 1. ○, Without ultraviolet light absorber; ●, with ultraviolet light absorber.

(9), in their investigations of fastness of dyes in textiles. As was indicated previously, the changes in reaction rates that are observed as the duration of irradiation is increased do not necessarily indicate that the fading mechanism consists of a series of consecutive reactions of different rates. Rather, they are generally due to several contributing factors, amongst which are: (*a*) alterations of the quantity of light reflected and absorbed at the surface of the tablet as fading proceeds, (*b*) subsequent changes in the amount of energy available to bring molecules to the excited state, (*c*) possible absorption of certain amounts of energy by the decomposition products, (*d*) porous nature of the tablets, (*e*) influence of substrates on the photoreaction, (*f*) the possibility of reaction between dye and its decomposition products, and (*g*) secondary reactions involving the dye and atmosphere.

Since the rate constants obtained from the first slope of the curve most accurately represent the photodegradation of the dye at the tablet surface, only the initial rate constants will be used in the subsequent discussions. The rate constants calculated from the initial slopes of the plots of log absorbance *vs.* time are summarized in Table I.

The data presented in this table demonstrate that the ultraviolet absorber exerted no significant protective action against fading from the surface of tablets colored with FD&C Red No. 3. However, for tablets colored with FD&C Yellow No. 5 the ultraviolet absorber reduced substantially the fading rate at the tablet surface. It is also apparent from the data in this table that the ultraviolet absorber was most effective against the light radiations from the sunlamp which was composed of significant energy in the near ultraviolet. This can be attributed to the fact that the ultraviolet absorber transmits essentially no light energy up to 400 mμ. The protective action of the ultraviolet absorber against ordinary and exaggerated room illumination was minor as compared to that observed under sunlamp illumination.

The radiations from the sunlamp caused the most rapid fading for each color studied. This may be explained by the fact that the radiations in the ultraviolet spectrum emitted by the sunlamp are more active chemically than the longer visible wavelength radiations.

It is important to note that the R.S. sunlamp radiates a substantial amount of heat as well as light radiations. In order to diminish the influence of heat on the rate of fading of the colored tablets, an exhaust fan was placed into the light cabinet. This reduced the temperature to a constant level of 40°. Thus, in evaluating the data obtained under this light source, one must realize that the fading is due to both light and thermal radiations. In the studies performed under normal and exaggerated room illuminations the temperature of the tablets was maintained at approximately 25°.

From the information obtained in this investigation, it is apparent that ultraviolet absorbing chemicals exert only small, if any, stabilizing effects against fading of colored tablets exposed to normal and exaggerated artificial room illumination. On the other hand, with respect to protection against sun radiations, ultraviolet absorbers can be very beneficial. In certain instances, however, the use of ultraviolet absorbers in the presence of sun radiations may not exert any protective action, as evidenced by the data obtained for tablets colored with FD&C Red No. 3. Hardy and Coleman (10) and Gantz and Sumner (11) also found that ultraviolet absorbers do not protect all dyestuffs.

SUMMARY

1. The photostabilizing effect of the ultraviolet absorber 2,4-dihydroxybenzophenone for tablets colored with three certified dyes was evaluated. These studies were performed under light sources simulating the spectral energy distribution of sunlight and normal and exaggerated room illumination.

2. The relative protective effect of this ultraviolet absorber for the three colors studied was as follows: FD&C Yellow No. 5 > FD&C Blue No. 1 > FD&C Red No. 3.

3. The complete absorption characteristics of the color must be studied when determining the factors responsible for the photostabilizing effects of the ultraviolet absorber.

4. The ultraviolet absorber was most effective against color fading for tablets exposed to simulated sunlight. However, it must be remembered that drugs are rarely exposed to direct or indirect sunlight.

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(2) U. S. pat. 2,664,383.
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(11) Gantz, G. M., and Sumner, W. G., *Textile Research J.*, **27**, 244(1957).

Color Stability of Tablet Formulations VI

Preliminary Study of Temperature Dependency of Colorant Loss in Tablets at Various pH Levels

By C. J. SWARTZ, L. LACHMAN, T. URBANYI, S. WEINSTEIN, and J. COOPER

The influence of temperature and pH on the surface color and total dye content of tablets colored with selected dyes was studied. The representative dyes included were FD&C Red No. 4, Blue No. 1, and Yellow No. 5. Samples of tablets colored with these dyes and buffered at several pH levels were stored at elevated temperatures and tested for both residual dye concentration and fading at their surface. FD&C Red No. 4 was found to be the most stable of the three dyes tested. However, from the results of this study there appears to be no correlation between the fading at the surface of the tablets and the loss in colorant concentration at elevated temperatures.

THE USE of accelerated stability tests for predicting the extended shelf life of pharmaceutical dosage forms has gained considerable acceptance in recent years. Through the application of chemical kinetic principles to elevated temperature storage data, it has been possible to predict accurately the stability of active ingredients at ambient temperatures.

In the past several years a number of reports have appeared in the literature relative to the thermal stability of certified colorants in liquid preparations (1–3). However, little information on the thermal stability of colorants in tablets has been reported. Since tablets constitute the major dosage form in most pharmaceutical companies, a study of color degradation in tablets was essential. Consequently, this study was initiated to evaluate the influence of thermal radiations on dyes when used in tablets.

Received April 28, 1961, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
Accepted for publication July 24, 1961.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.

EXPERIMENTAL

Materials Used.—FD&C Blue No. 1, FD&C Yellow No. 5, and FD&C Red No. 4, H. Kohnstamm and Co. 0.2 *M* Tartrate buffer at pH 3. 0.2 *M* Phosphate-citrate buffer at pH 5. 0.2 *M* Phosphate-tartrate buffer at pH 7.

Equipment.—Precision and Hotpack ovens. Comprex tablet press. Beckman spectrophotometer model DU with reflectance attachment (4). Cary

SUMMARY

1. The photostabilizing effect of the ultraviolet absorber 2,4-dihydroxybenzophenone for tablets colored with three certified dyes was evaluated. These studies were performed under light sources simulating the spectral energy distribution of sunlight and normal and exaggerated room illumination.

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Equipment.—Precision and Hotpack ovens. Comprex tablet press. Beckman spectrophotometer model DU with reflectance attachment (4). Cary

recording spectrophotometer model 11. Beckman Zeromatic pH meter. Fitzpatrick Homoloid mill. Beckman Aquameter.

Preparation of Tablets.—Tablets containing the water-soluble dyes were prepared unbuffered and at the three pH levels: 3, 5, 7. The tablet formulations prepared for this study were as follows

	Unbuffered, %	pH 3, %	pH 5, %	pH 7, %
Calcium sulfate $\cdot 2H_2O$	96.90	93.42	91.98	89.17
Tragacanth U.S.P.	2.00	2.00	2.00	2.00
Magnesium stearate U.S.P.	1.00	1.00	1.00	1.00
Dye	0.10	0.10	0.10	0.10
Tartaric acid	...	0.75	...	...
Sodium tartrate $\cdot 2H_2O$	...	2.73	...	2.73
Sodium phosphate, primary	...	...	1.58	...
Sodium citrate	...	...	3.34	...
Sodium phosphate, dibasic $\cdot 7H_2O$	...	...	...	5.00
Purified water	*q.s.*	*q.s.*	*q.s.*	*q.s.*

The granulations were made by dissolving the buffer salts in 150 ml. of water at 80° and the dye in 50 ml. of water at 40°. The dye solution was added first to the mixed powders, followed by the buffer solution. The separate addition of the dye and buffer solutions was necessary in order to prevent precipitation of the dye by the buffer salts. All granulations were terminally milled through a Fitzpatrick Homoloid mill using a 050 screen to insure uniform color distribution in the finished tablet. The tablets were compressed on a Comprex tablet press using flat faced $^{8}/_{32}$ inch punches to a hardness of 7 Kg./in.^{2} with a weight of 100 mg. The moisture content of the dried granulations was maintained below 0.5%. The use of the dyes at the 0.1% concentration level was necessary so that sufficient dye would be available to permit accurate extractions for quantitative determination.

Storage of Samples.—Tablets colored with FD&C Red No. 4, Blue No. 1, and Yellow No. 5 were studied in such a manner that light or moisture-induced effects on the color degradation would be kept to a minimum. Indicating Drierite, 100 Gm., was placed into 8-oz. wide-mouth amber bottles which had been previously covered with opaque masking tape. The Drierite was covered with glass wool and 1,000 tablets were added to each bottle and the bottles capped. Sample bottles were stored at 25, 60, and 80 ± 1°. Test samples were withdrawn at designated time intervals for chemical assay and reflectance measurements.

Measurement of Tablet Reflectance.—The techniques and equipment employed to follow changes in the reflectance at the surface of the tablets were described in a previous publication (4). Reflectance measurements were made on the colored tablets at 630 mμ for FD&C Blue No. 1, at 505 mμ for FD&C Red No. 4, and at 430 mμ for FD&C Yellow No. 5.

Spectrophotometric Analysis for Dye Content of Tablets.—The changes in dye concentration with storage were determined in accordance with the following procedure: An adequate number of tablets were ground in a mortar from which a 1.0-Gm. aliquot was accurately weighed and transferred into a glass-stoppered 40-ml. centrifuge tube. The sample was extracted with two 25-ml. portions of methanol followed by two 20-ml. portions of water. Prior to the first aqueous extraction, 5 Gm. of sodium chloride was added to each centrifuge tube. Each extract was filtered through a medium porosity sintered-glass filter into a 100-ml. volumetric flask containing an additional 5 Gm. of sodium chloride. The sodium chloride was employed to coagulate the colloidal tragacanth present in the extract. Following thorough agitation, the solution was brought to 100 ml. with water. A quantity of this solution was passed through a medium sintered-glass filter and complete absorption curves in the visible spectrum were run on the Cary spectrophotometer. As a result of the intense absorption of FD&C Blue No. 1, only 0.5-Gm. samples of tablets containing this dye were used.

Determination of Tablet pH.—Ten tablets were crushed in a mortar and triturated with 10 ml. of purified water. The material was then transferred to a 20-ml. beaker and stirred for 5 minutes. The pH of the suspension was determined, using the Beckman Zeromatic pH meter. The results are recorded in Table I.

TABLE I.—APPARENT pH OF SUSPENSIONS OF CRUSHED TABLETS IN WATER

pH	FD&C Red No. 4	FD&C Yellow No. 5	FD&C Blue No. 1
Unbuffered	6.4	5.9	6.5
3	3.0	3.1	3.0
5	4.9	5.1	5.2
7	6.8	6.6	6.5

RESULTS AND DISCUSSION

The influence of temperature and pH on the thermal stability of colorants in tablets has been investigated. In addition to studying the changes that take place in dye concentration, observations of fading at the tablet surface were made.

A representative plot showing the decrease in the concentration of FD&C Yellow No. 5 stored at 80° for 60 days is shown in Fig. 1. It is evident from this figure that the absorption decreases with storage. The complete curves, however, maintain their characteristics at all the temperature and pH levels tested. The tablets buffered to pH 7 showed the greatest loss of colorant. The curves for the unbuffered and pH 3 tablets have not been presented here, as no breakdown was observed under these conditions.

The per cent total loss of colorants after storage for 60 days at 25, 60, and 80° is summarized in Table II. It is apparent from this data that the tablets colored with FD&C Red No. 4 have exhibited the greatest stability under the conditions of study. Only the tablets buffered at pH 5 showed any loss of

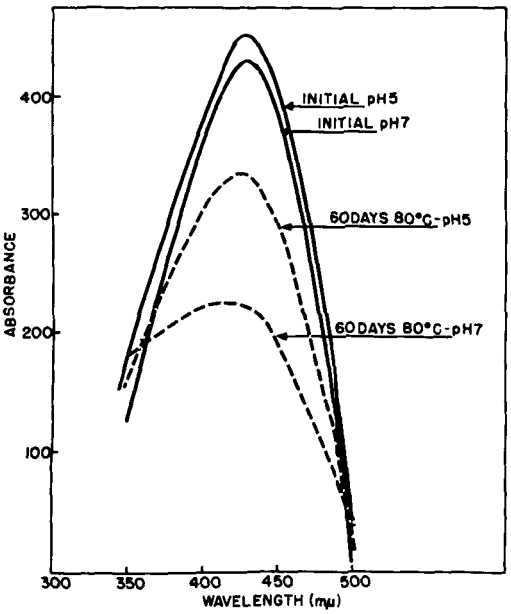


Fig. 1.—The effect of temperature, 80°, on the visible absorption spectra of FD&C Yellow No. 5 dye extracted from tablets.

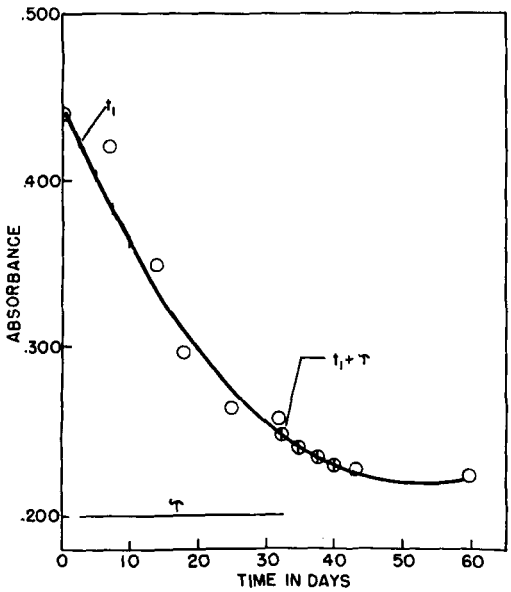


Fig. 2.—Plot of change of absorbance of FD&C Yellow No. 5 at 80°, pH 7, with time.

dye content. In the case of tablets colored with FD&C Yellow No. 5, poor stability is noted in the tablets buffered to a pH of 5 and 7, with the tablets at pH 7 showing approximately twice the loss of those at pH 5. This may be attributed to the action of the buffer salts and/or pH environment created by them. The data for the tablets colored with FD&C Blue No. 1 indicate that loss of dye concentration is essentially pH independent. The per cent loss in dye concentration both at 60 and 80° are of a similar magnitude. It should be noted that at 25° no change in dye concentration resulted for the tablets colored with the three dyes and at the several pH levels.

A representative plot for the change in absorbance with time for the tablets colored with FD&C Yellow No. 5 is shown in Fig. 2. It is evident from this curve that the reaction proceeds to an infinity value. In order to obtain an accurate estimate of the rate of loss of color from the tablets, the loss data was treated according to the method of Guggenheim (5). By employing this technique it is unnecessary to know the value at infinity time. The method is applicable where a property other than concentration is being followed. If t_1, t_2, t_3, and t_4 and $t_1 + \tau$, $t_2 + \tau$, $t_3 + \tau$, and $t_4 + \tau$, are selected where τ is a constant increment, the differential equation for a first-order reaction takes the form

$$\ln (P_t - P_{t+\tau}) + kt = \text{constant}$$

By plotting $\log (P_t - P_{t+\tau})$ *vs.* t, the slope of the line will be equivalent to $-k/2.3$. Therefore, by measuring the slope of this line, one is able to obtain the first-order rate constant for the reaction ($k = -\text{slope} \times 2.3$). The interval τ should be at least two or three times as great as the half-life period of the reaction in order to obtain maximum accuracy.

By the application of the Guggenheim method, the rate constants (k) for loss in colorant concentration were obtained for tablets colored with FD&C Yellow No. 5 at pH 5 and 7 stored at 60 and 80°.

At pH 5: 60° = 5.6×10^{-2} days $^{-1}$; 80° = 12.7×10^{-2} days^{-1}. At pH 7: 60° = 1.43×10^{-2} days^{-1}; 80° = 4.1×10^{-2} days^{-1}.

It is interesting to note that although the samples buffered at pH 5 are degrading at a faster rate, these tablets show only about one-half the loss in total dye concentration when compared with the tablets buffered to pH 7 (Table II). This is due to the fact that the loss curve has a higher infinity value for the tablets buffered to a pH 5 than those at pH 7, thus a lower net rate results. But the rate at which the loss in color reaches the infinity value at pH 5 is faster than that at pH 7 and, according to chemical kinetic principles, the rates obtained here must be used.

In Table III a summary is presented of the surface fading of tablets colored with FD&C Yellow No. 5 and Blue No. 1 and at several pH levels. Uncolored tablets were used as a control and reflectance readings obtained from the surface of these tablets, after storage, were subtracted from the reflectance values obtained with the colored tablets after similar storage. These results show that the fading from the surface of the tablets colored with FD&C Blue No. 1 was small and in the same range for all pH levels. However, for the tablets colored with FD&C Yellow No. 5, a significant surface color change was observed in all the buffered samples. Although there appears to be considerable surface fading for the tablets at pH 3, the color concentration loss obtained at this pH was not of the same order.

As a result of irregularities in the surface hue of tablets colored with FD&C Red No. 4, reflectance data for these tablets have been omitted. It is apparent from the information presented in Tables II and III that there appears to be no correlation between the fading at the surface of the tablets and the loss in colorant concentration. A possible

TABLE II.—PER CENT LOSS OF DYE IN BUFFERED TABLETS AFTER 60 DAYS STORAGE AT VARIOUS TEMPERATURES

Temperature, °C.	FD&C Red No. 4				FD&C Yellow No. 5				FD&C Blue No. 1			
	Unbuffered	pH 3	pH 5	pH 7	Unbuffered	pH 3	pH 5	pH 7	Unbuffered	pH 3	pH 5	pH 7
25	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	15	29	11	11	13	8
80	0	0	6	0	0	0	25	48	13	11	19	12

TABLE III.—REFLECTANCE CHANGES AT THE SURFACE OF TABLETS FOLLOWING STORAGE

	FD&C Blue No. 1				FD&C Yellow No. 5			
	Unbuffered	pH 3	pH 5	pH 7	Unbuffered	pH 3	pH 5	pH 7
Initial	1.200	1.120	1.045	1.080	0.825	0.768	0.785	0.770
25°, 60 days	1.175	1.110	1.020	1.030	0.825	0.760	0.760	0.750
80°, 60 days	1.000	0.884	0.851	0.885	0.715	0.500	0.515	0.475

explanation for this is the high concentration of dye used for the tablets.

The pH of the colored and uncolored tablets showed similar variations relative to maintenance of initial pH with storage. A slight decrease in pH was noticed in all cases except for the tablets buffered at pH 3 where essentially no change took place. Initially, it was intended to utilize the same buffer salts (phosphates) for the entire pH range of this investigation; however, it soon became apparent that the buffer capacity of the phosphate salts was not adequate at all the pH levels required. Therefore, varied buffered salt combinations were used for the different pH's to give optimum buffer capacity.

This study was designed to obtain an initial insight into the thermal stability of colorants when used in tablets. Although only limited data have been obtained, further experiments are necessary to define the complex factors contributing to dye instability. As a result of this information, additional investigations are contemplated to evaluate thoroughly the thermal stability of all FD&C colorants when employed in solid dosage forms.

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Stability of Metal Complexes of Nuclear-Substituted Salicylic Acids: Correlation with Biological Effects

By WILLIAM O. FOYE and JOSEPH G. TURCOTTE

Stability constants are reported for the chelation of ferric and aluminum ions by a series of salicylic acid derivatives having amino, alkyl, chloro, and nitro groups in the ring. The sequence of complex stabilities found is in agreement with current organic theory and shows a parallelism with the ionization constants. Three of the biological effects known for salicylates, antibacterial, analgesic-antipyretic, and fungicidal, can be related to the magnitude of complex stability or ionization constant.

A NUMBER of experimental approaches have been made in the attempt to explain the biological effects of the salicylates. One explanation for these effects has implicated the metal-binding ability of the salicylates (1–4), since the *meta*- and *para*-hydroxybenzoic acids, which are incapable of binding metal ions through complexation, exert none of the classical salicylate effects (1). The literature, however, does not provide much information regarding the relative abilities of the various salicylates to bind metal ions. The measurement of stability constants should provide a much fuller knowledge of these abilities and give us a better insight as to the importance of metal-binding in salicylate actions.

Received April 28, 1961, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.
Accepted for publication June 26, 1961.
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TABLE I.—KNOWN STABILITY CONSTANTS FOR VARIOUS SALICYLATES AND METAL CATIONS (Water, 20°)

Ligand	Cation: Fe^{+++} log K'	Fe^{+++} log K''	Cu^{++} log K'	Cu^{++} log K''	Fe^{++} log K'	Fe^{++} log K''	Ref.
Salicylic acid	16.35	11.90	10.60	7.85	6.55	4.7	(6)
5-Sulfosalicylic acid	14.60	10.55	9.50	6.80	5.90	<4.0	(6)
5-Aminosalicylic acid	16.97	12.04	..	..	..	..	[a]
Methyl salicylate	..	..	5.90	..	9.74[a]	..	(6)
Salicyl aldehyde	..	..	5.75	..	..	..	(6)
Gentisic acid	10.8	8.0	7.0	4.9	..	..	(7)
Salicyluric acid	9.0	7.0	6.1	5.6	..	..	(7)
Salicylamide	10.02	6.24	..	..	..	..	[a]

[a] Ågren, A., *Acta Chem. Scand.*, **9**, 39(1955).

Stability constants for the chelation of biologically important metal ions by salicylic acid (5–7), as well as those of a few salicylic acid derivatives, have been recorded. The latter include methyl salicylate, salicylamide, 5-sulfo- and 5-aminosalicylic acids, gentisic acid, and salicyluric acid. The constants for these substances with pertinent metal ions are listed in Table I.

The literature provides ample indication that salicylic acid or its derivatives become involved in enzymatic reactions, for which temporary binding of the prosthetic metal is a likely possibility. Among the more recent reports of this nature are those of Piccinelli (8), in which sodium salicylate was found to cause marked change in sugar metabolism; Messer (9), who found that sodium salicylate and sodium acetylsalicylate inhibit the synthesis of glutamine; Adams (10), who found a correlation between anti-inflammatory activity, increase in oxygen consumption, and uncoupling of oxidative phosphorylation; Durham (11), who showed that *p*-aminosalicylic acid antagonized the oxidative dissimilation of *p*-aminobenzoic acid in flavobacteria; and Janota (12), who reported that prolonged administration of salicylates increased the activity of plasma enzymes in rabbits. Direct inhibition of a copper oxidase, tyrosinase, by salicylic acid is also known.[1]

Stability constants have now been determined for the reaction with metal ions of a series of salicylic acids, having various substituents in the 3, 4, and 5 positions of the ring, with the expectation that a correlation might be found between either strength or weakness of metal-binding and a salicylate action. Where a temporary combination with the constituent metal of an enzyme might take place, as previously postulated (13), a lowering of stability constant (from that of salicylic acid with metals) should indicate more effective compounds. That these substituents might affect other physical properties of the molecules, such as solubility characteristics, is presently under consideration.

[1] C. W. Bauer, personal communication.

The metal ions selected for this experiment were ferric and aluminum. Ferric ion was considered more appropriate than cupric, for instance, since Perrin's calculations (6) revealed that salicylic acid has a stronger affinity for ferric ion than has glycine, a representative cellular ligand, by a factor of 400:1, whereas glycine has the stronger affinity for cupric ion, by a factor of 45:1. Aluminum was selected as a suitable trivalent ion for comparative purposes. The possibility of the same chelating strength sequence for two metal ions could then be determined.

METHODS

Materials.—The metal salts used were Baker analyzed reagents $FeCl_3 \cdot 6H_2O$ and $AlCl_3 \cdot 6H_2O$. The organic ligands selected for study (listed in Table II) were commercially available with the exception of the diisopropyl and *tert*-butyl derivatives of salicylic acid which had to be prepared. The ligands obtained commercially were purified by several recrystallizations from aqueous alcohol and charcoal. Purity was confirmed by melting points and paper chromatography using butanol saturated with ammonia.

Carbonate-free 0.1000 *N* sodium hydroxide solution was prepared from a concentrated solution of sodium hydroxide U.S.P. appropriately diluted with recently boiled and cooled distilled water. It was treated with saturated barium hydroxide solution for 12 hours, filtered, and standardized.

TABLE II.—IONIZATION CONSTANTS (20°)

Compound	pKa′ ($-CO_2H$)	pKa ($-OH$)
4-Aminosalicylic acid	6.75	13.33
4-Methylsalicylic acid	5.72	13.32
3-Methylsalicylic acid	5.70	13.30
3,5-Diisopropylsalicylic acid	5.92	13.28
5-*tert*-Butylsalicylic acid	5.64	13.20
3-Isopropyl-6-methylsalicylic (*o*-thymotic) acid[a]	5.60	13.21
Salicylic acid	5.57	13.14
5-Chlorosalicylic acid	4.85	13.34
5-Nitrosalicylic acid	3.92	12.83
3-Nitrosalicylic acid	3.81	12.77

[a] Generously supplied by Dr. Paul J. Jannke, University of Connecticut, Storrs.

TABLE III.—STABILITY CONSTANTS OF THE SALICYLATE COMPLEXES (20°)

Ligand	Fe^{+++} log K'	Fe^{+++} log K''	Fe^{+++} log Ks[a]	Al^{+++} log K'	Al^{+++} log K''	Al^{+++} log Ks[b]
4-Aminosalicylic acid	16.30	13.91	30.48	15.57	12.45	28.02
4-Methylsalicylic acid	16.26	11.89	29.84	15.76	11.65	27.41
3,5-Diisopropylsalicylic acid	16.23	11.59	29.66	15.62	11.75	27.37
5-*tert*-Butylsalicylic acid	16.06	12.21	29.44	15.60	11.56	27.16
Salicylic acid	16.53	12.69	29.34	15.62	11.45	27.07
3-Isopropyl-6-methylsalicylic acid	16.03	11.34	29.08	15.45	11.50	26.95
3-Methylsalicylic acid	15.99	11.85	29.04	15.26	11.53	26.79
5-Chlorosalicylic acid	16.07	12.82	28.96	16.02	11.73	27.75
3-Nitrosalicylic acid	14.60	12.46	27.54	14.25	11.85	26.10
5-Nitrosalicylic acid	14.51	12.67	26.86	14.31	12.16	26.47

[a] Calculated from $\log Ks = -2 \log (Sc)$, when $\bar{n} = 1$. [b] Calculated from $Ks = \log K' + \log K''$.

Preparation of 3,5-Diisopropylsalicylic Acid.—The procedure of Meyer and Bernhauer (14) for 5-isopropylsalicylic acid was used with modifications. A 1,600-ml. portion of 80% sulfuric acid, 60 Gm. (0.43 mole) of salicylic acid, and 40 ml. of 2-propanol were stirred rapidly at 70° for 1 hour. The flocculant product was isolated and recrystallized from glacial acetic acid. A yield of approximately 80% of white, crystalline material was obtained which melted at 117–118°. Literature values for the melting points of both the 5-isopropyl and 3,5-diisopropyl derivatives were found to be in the range 116–118°.

Anal.[2]—Calcd. for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16. Found: C, 70.90; H, 8.20.

Preparation of 5-*tert*-Butylsalicylic Acid.—Use of the method of Meyer and Bernhauer (14) was found to give a sulfonated product, so it was modified as follows. Sixty grams (0.43 mole) of salicylic acid, 75 ml. of *tert*-butyl alcohol, and 1,600 ml. of 80% sulfuric acid were stirred for 1 hour, during which time the temperature was raised to 75°. The resulting solution was allowed to cool overnight, charcoal was added, and the mixture was boiled for 5 minutes. It was filtered and allowed to cool, and the white, crystalline product was isolated and recrystallized from dilute acetic acid; m.p. 151–152°.

Anal.—Calcd. for $C_{11}H_{14}O_3$: C, 68.02; H, 7.26. Found: C, 68.00; H, 7.30.

Ionization Constants.—Values for primary and secondary ionization constants for each compound were determined by the potentiometric procedure of Osol and Kleckner (15), using 0.01 *M* solutions of the salicylates in 95% ethanol. A Beckman model E-2 electrode sensitive over the entire pH range was employed to give more accurate constants for the phenolic group. Table II lists the constants obtained.

Titrations.—The titrations were carried out under nitrogen as previously described (7) except that 0.01 *M* solutions of the ligands were made in 95% ethanol instead of in water. Aqueous base was used as before for titration, and the same volumes and concentrations were used for the determination of ionization constants as for the stability constants. The final solutions in each case contain approximately 79% alcohol. A Beckman Zeromatic pH meter with a Beckman model E-2 electrode was employed. Titrations were repeated for each system until pH values agreed to within 0.02–0.05 units for each run. A Friden fully automatic calculator, model SRW, was used for most of the calculations, which were carried out as previously described (7). The constants obtained are listed in Table III.

[2] Carbon-hydrogen analyses were done by Carol K. Fitz, Needham, Mass.

RESULTS AND DISCUSSION

In Table III are listed the values calculated for the first and second stability constants (log K' and log K'') of the metallic complexes, which correspond to the extent of formation of those having 1:1 and 2:1 ligand-metal ratios. In some cases, precipitation of the complexes prevented the determination of the log K''' values, and in other cases the values obtained were of doubtful accuracy. For purposes of comparison, "log Ks" values are listed, which indicate the overall stability of complex formation through the 2:1 complex stage. It would be unlikely that values for the third stability constant would alter the sequence of chelating abilities as shown. Therefore the log Ks values for the first and second stability constants, calculated by two different procedures (that one being listed that gave the greatest spread of values in each case) should be adequate for comparison.

The sequence of chelating abilities found was essentially the same for both the Fe^{+++} and Al^{+++} complexes. This sequence is also in agreement with that determined for copper complexes of substituted salicylaldehydes by Calvin and Wilson (16). The results reported for the salicylaldehydes in general resemble those reported here for the substituted salicylates: there was but a small variation in stability constants among the alkyl derivatives which in general were slightly higher than the value for salicylaldehyde itself, the halogen-substituted salicylaldehydes gave somewhat lower values, and the 3- and 5-nitro derivatives gave the lowest constants. No amino derivatives were included.

Comparison of the stability constants of the complexes with the ionization constants of the acids shows a rough parallelism between acidity (of both carboxyl and phenolic groups) and complex stability. One immediately questions the position of the 5-chlorosalicylic acid complexes in the stability sequence, since its phenolic ionization constant would indicate a stability comparable to that of the 4-amino- or 4-methylsalicylic acids, while its carboxyl ionization constant would place it just above the nitrosalicylic acids in the stability sequence. That the stability of the ferric complex falls between the values for the ferric complexes of the alkyl

and nitro acids indicates that the acidity of the carboxyl group contributes fully as much to the complex stability as the acidity (or basicity) of the phenolic group. However, with the aluminum complex of 5-chlorosalicylic acid, the stability of the complex is almost as great as that of the 4-amino derivative. In both cases, the value of log K' approaches that for the more stable complexes, as might be predicted from the value of the phenolic ionization constant.

The stability of the 3-methylsalicylic acid complexes is also seen to be out of position with regard to the ionization constants. In this case, a possible explanation is suggested immediately in steric hindrance which would tend to lower the stability constant to its observed value. That the 3,5-diisopropyl derivative does not produce the same demotion of stability constant may be due to the lesser numbers of α-hydrogen atoms in the 3-substituent.

A conclusion regarding the electronic effects of the substituents on complex stability can be readily seen from the constants reported here and is consistent with current organic chemical theory. Those groups capable of contributing electrons to the ring increase the basicity of the phenolic group and thus increase complex stability, whereas those groups that attract electrons decrease the phenolic basicity and complex stability as well. The 4-substituents apparently increase the basicity of the carboxyl function thus supporting the structure of the free chelating species shown in the previous paper (7). Similar observations regarding the effect of ionization constant on complex stability have been made before (7, 17).

A complete understanding of the role that complex stability plays in the biological effects of salicylates probably cannot be reached until the limiting effects of other properties, such as liposolubility, are known. However, the possibility may be advanced that the analgesic effects of salicylates do not require a high stability for possible metal complexes, whereas the antibacterial ability does. This may be concluded from the high stability noted for the complexes of 4-aminosalicylic acid, the only effective antitubercular agent among the salicylates, and the lower stability noted for the complexes of *o*-thymotic acid, reported to have a greater analgesic effect than has aspirin (18). The cresotinic acids (3-, 4-, and 5-methylsalicylic acids) have approximately the same antirheumatic efficacy as salicylic acid (19), and 4,5-dimethylacetylsalicylic acid has a similar antipyretic ability to that of aspirin (20). These observations are all in accord with the similar constants found for salicylic acid and its alkyl derivatives. It appears doubtful that the fungicidal activity of the salicylates is related to metal binding, since the most effective derivatives, the salicylanilides and alkyl ethers (21) would not be expected to bind metal ions to an appreciable extent.

SUMMARY

1. The avidities of a series of salicylic acids, having various substituents in the ring, for ferric and aluminum ions have been measured potentiometrically and recorded as stability constants.

2. The sequence of complex stabilities found with both metal ions is in agreement with that predicted from the electron-attracting and -repelling effects of the substituents, and shows a parallelism with the ionization constants of the ligands.

3. A relation between the magnitude of the complex stability constant and three of the biological effects of the salicylates has become evident. This has made possible the classification of these biological effects in terms of a physical constant (either complex stability or ionization constant). Antibacterial ability apparently requires high constants, analgesic-antirheumatic effects appear in compounds having constants similar to those of salicylic acid itself, and fungicidal ability has been found in those salicylates having no or very low metal-binding strength.

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Triphenyltetrazolium Determination of 17,21-Dihydroxy-20-ketosteroids in Tablets

By JOHN J. CALLAHAN, FRANK LITTERIO, ELI BRITT, BERNARD D. ROSEN, and JOHN OWENS

The recommended color development conditions of Johnson, King, and Vickers (1) for the triphenyltetrazolium determination of bulk hydrocortisone have been applied to the tablet dosage forms of prednisone and prednisolone.

THE U.S.P. XVI method (2) for the assay of the 17,21-dihydroxy-20-ketone side chain of steroids is the Mader-Buck (3) procedure. During the application of this method to tablets, it was noted that the variations obtained were greater than might have been anticipated. The recent publication of Johnson, King, and Vickers (1) using hydrocortisone bulk powder had conclusively demonstrated some of the sources of variance and had outlined a procedure to overcome them. The utilization of the Johnson, King, and Vickers color development conditions to the tablet dosage form of 17,21-dihydroxy-20-ketosteroids therefore seemed desirable.

EXPERIMENTAL

Reagents.—U.S.P. 95% ethanol. Triphenyltetrazolium chloride reagent solution; dissolve 50 mg. of 2,3,5-triphenyltetrazolium chloride in 10 ml. of U.S.P. ethanol, this solution should be freshly prepared and kept protected from light. Tetramethylammonium hydroxide reagent solution; dilute 5 ml. of 10% aqueous solution to 100 ml. with U.S.P. ethanol. Chloroform. Dry nitrogen.

Tablet Extraction.—The steroid is extracted from the tablets by suspending a crushed aliquot of the tablets in water and shaking with multiple portions of chloroform as directed in U.S.P. XVI. A 10-ml. aliquot of the chloroform extract is evaporated in a 50-ml. nitrogen-flushed low-actinic glass volumetric flask (or a glass-stoppered Erlenmeyer flask) under a stream of nitrogen, on a steam bath. Heating with nitrogen is continued for 3 minutes after dryness is observed.

Color Development.—After the addition of 20 ml. of U.S.P. alcohol, the stoppered flask is mechanically shaken for 15 minutes, 2 ml. of triphenyltetrazolium chloride reagent solution is added, followed by 2 ml. of tetramethylammonium hydroxide reagent solution. The surface of the solution is swept with nitrogen for 15 seconds, the flask immediately stoppered, and placed in a 30° water bath. The reaction is allowed to proceed for 45 minutes for prednisone (Meticorten) tablets and 60 minutes for prednisolone (Meticortelone) tablets. The absorbance at 485 mμ is measured against a reagent blank.

Received May 16, 1961, from Schering Corp., Bloomfield, N. J.
Accepted for publication June 20, 1961.

RESULTS AND DISCUSSION

The application of the U.S.P. XVI procedure for the assay of 17,21-dihydroxy-20-ketosteroids has resulted in a large variation both in the results obtained in any given day as well as those obtained from day to day. Very poor reproducibility is often obtained in duplicate determinations of the standards themselves. Table I shows the results obtained by two analysts on five batches of tablets assayed on five consecutive days.

The major differences between U.S.P. XVI and the Johnson, King, and Vickers procedure are the use by the latter of low actinic glassware, removal of air in the reaction flasks by nitrogen, use of a 30° bath for the color development, and the reversal of the order of addition of the two reagents, tetramethylammonium hydroxide and triphenyltetrazolium chloride.

Before proceeding with the tablet assays, the effect of the variables studied by Johnson, *et al.*, were re-examined using prednisone and prednisolone standards.

Effect of Alcohol.—Anhydrous ethanol and 95% ethanol are the solvents used in most published procedures (1–5). The effects of different alcohols both on the steroid and on the reagent blanks are shown in Table II. It is not known whether the exceedingly high values of the blank readings obtained with isopropanol and *t*-butanol are due to the alcohols themselves or to some possible contaminant in them. Ethanol is the best alcohol to use in this procedure.

Effect of Water.—Increasing the water content in ethanol decreased the rate of color development. However, when longer development times were used, the absorbance readings were comparable to those obtained with the anhydrous ethanol. Consequently, the use of U.S.P. 95% ethanol was found to be very satisfactory.

Effect of Contaminants.—Low absorbance values were obtained in the presence of air, oxygen, and acetic acid. If these were present in relatively large amounts, the color development was inhibited. The presence of small amounts of chloroform and benzene did not interfere with the reaction Small amounts of acetaldehyde increased the absorbance readings.

Effect of Tetramethylammonium Hydroxide Concentration.—Both the U.S P. XVI and the Johnson, King, and Vickers procedures specify the same concentration of tetramethylammonium hydroxide. When this concentration was halved, the rate of color formation was identical but higher absorbance

TABLE I.—ANALYSIS OF PREDNISOLONE TABLETS BY THE U.S.P. XVI PROCEDURE[a]

Sample	Absorbance											
	Analyst A						Analyst B					
	Std. 1	Std. 2	Tab. 1		Tab. 2		Std. 1	Std. 2	Tab. 1		Tab. 2	
A	0.403	0.381	0.377	0.350	0.363	0.368	0.394	0.392	0.382	0.403	0.388	0.400
B	0.383	0.388	0.348	0.345	0.357	0.355	0.400	0.405	0.348	0.357	0.367	0.370
C	0.390	0.373	0.425	0.422	0.378	0.374	0.395	0.383	0.382	0.386	0.386	0.387
D	0.403	0.380	0.387	0.383	0.390	0.387	0.410	0.409	0.399	0.408	0.411	0.408
E	0.381	0.390	0.393	0.395	0.395	0.394	0.403	0.404	0.411	0.415	0.402	0.406

[a] Steroid concentration = 5 mg. per tablet.

TABLE II.—EFFECT OF VARIOUS ALCOHOLS ON ABSORBANCE VALUES[a]

Alcohol	Absorbance	
	Blank	Steroid
Anhydrous ethanol	0.050	0.470
S. D. 3A, 95% ethanol	0.045	0.430
U.S.P. 95% ethanol	0.040	0.407
Methanol	0.010	0.090
Isopropanol	0.590	0.700
t-Butanol	0.820	1.510

[a] Prednisone concentration = 0.25 mg. per 24 ml.

TABLE III.—EFFECT OF BASE STRENGTH ON THE RATE OF COLOR DEVELOPMENT[a,b]

Development, min.	Absorbance	
	Base 1:10 Dilution	Base 1:20 Dilution
15	0.411	0.410
30	0.408	0.420
45	0.410	0.425
60	0.407	0.420
75	0.402	0.420

[a] Prednisone concentration = 0.25 mg. per 24 ml. [b] Temperature for color development was 25°.

TABLE IV.—EFFECT OF TIME BETWEEN REAGENT ADDITIONS[a]

Time Between Additions, min.	Absorbance	
	Base[b] Added First	Stain[c] Added First
0.5	0.400	0.340
5	0.310	0.380
10	0.272	0.370
15	0.214	0.365
20	0.180	0.380

[a] Prednisone concentration = 0.25 mg. per 24 ml. [b] Tetramethylammonium hydroxide. [c] Triphenyltetrazolium chloride.

values were obtained as well as slightly improved color stability as shown in Table III.

Order of Reagent Addition.—The order of addition of tetramethylammonium hydroxide reagent solution and triphenyltetrazolium chloride reagent solution was relatively unimportant when only a few samples were run. However, when multiple samples were run, such as is usual in a quality control laboratory, the addition of one reagent to all the reaction flasks before the next reagent addition may introduce a 5 to 10-minute interval between the addition of the tetramethylammonium hydroxide and the triphenyltetrazolium chloride. In this case, the order of reagent addition becomes very important. If triphenyltetrazolium chloride was added first to each reaction flask, no difficulty was encountered. However, if the tetramethylammonium hydroxide was added first, drastic reductions in absorbance values were encountered as the time interval between reagent additions was increased (Table IV), when using identical color development times measured from the time of addition of the last reagent.

TABLE V.—EFFECT OF TIME ON COLOR STABILITY[a,b]

Time, min.	Absorbance	
	Prednisone	Prednisolone
15	0.415	0.310
30	0.430	0.430
60	0.423	0.455
90	0.425	0.455
120	0.422	0.455

[a] Steroid concentration = 0.25 mg. per 24 ml. [b] 30° water bath, U.S.P. 95% ethanol, and 1:20 base dilution used.

TABLE VI.—EFFECT OF NITROGEN FLUSHING PRIOR TO COLOR DEVELOPMENT[a]

Experiment	Absorbance	
	Standard	Tablet
A[b]	0.387	0.420
B[c]	0.399	0.402
C[d]	0.428	0.430

[a] Prednisolone concentration = 0.25 mg. per 24 ml. [b] Chloroform evaporated under nitrogen but standard and blank flasks were not nitrogen flushed. [c] Chloroform evaporated in air. Standard and blank flasks were not flushed with nitrogen. [d] Chloroform evaporated under nitrogen. Standard and blank flasks were also flushed with nitrogen.

TABLE VII.—EFFECT OF TIME ON COLOR STABILITY OF TABLET EXTRACT[a]

Time, min.	Absorbance	
	Standard	Tablet Extract
30	0.442	0.443
60	0.441	0.441
90	0.435	0.439

[a] Prednisone concentration = 1.0 mg. per tablet.

Effect of Temperature on Color Development.—The rate of color development increases with temperature. Some fluctuations in assay results were obtained when (room) temperature fluctuations were present. For this reason, it was found expedient to adopt a standardized bath temperature of 30° as recommended by Johnson, King, and Vickers (1).

Color Stability.—When the stated precautions were observed, the stability of the developed color was very good. Almost no drop in absorbance was found after 2 hours in the 30° water bath, as shown in Table V.

Tablet Extract Preparation.—It was found that improper evaporation of the aliquot of the chloro-

TABLE VIII.—ANALYSIS OF PREDNISOLONE TABLETS BY THE MODIFIED PROCEDURE[a]

	Absorbance											
	Analyst A						Analyst B					
Sample	Std. 1	Std. 2	Tab. 1		Tab. 2		Std. 1	Std. 2	Tab. 1		Tab. 2	
A	0.465	0.472	0.442	0.440	0.441	0.435	0.458	0.466	0.450	0.450	0.460	0.460
B	0.464	0.466	0.403	0.406	0.404	0.402	0.458	0.466	0.433	0.430	0.432	0.433
C	0.465	0.465	0.422	0.423	0.454	0.458	0.467	0.468	0.430	0.432	0.452	0.452
D	0.462	0.462	0.452	0.450	0.470	0.469	0.467	0.468	0.452	0.454	0.462	0.464
E	0.465	0.465	0.450	0.462	0.440	0.434	0.458	0.466	0.448	0.453	0.456	0.456

[a] Steroid concentration = 5 mg. per tablet.

form extract of the tablet led to variable results if the higher boiling residues in the chloroform were not removed. This was easily effected by further heating the reaction flasks at about 95° under a stream of nitrogen for about 3 minutes longer after the flasks appeared to be dry. The flasks in which the standard and reagent blank were run also had to be flushed with nitrogen while still empty. This was found to be necessary because a comparison of the absorption readings of the solutions (obtained from the flasks nitrogen flushed and not nitrogen flushed at the start) indicated that higher absorbance values were obtained for the flushed flasks, as shown in Table VI.

It was also found that nitrogen flushing all the flasks while empty (as in the cases where chloroform was evaporated, just after the 3-minute extra heating time but before the addition of the alcohol) and nitrogen flushing again after all the reagents had been added was just as effective as the Johnson, King, and Vickers procedure of nitrogen flushing after each addition of reagent.

Tablet Extract Color Stability.—Table VII shows the rate of color development for prednisone extracted from tablets compared to standard prednisone. No substantial difference was found when ethanol U.S.P., 0.5% tetramethylammonium hydroxide concentration, and a 30° bath were used.

Tablet Analysis.—The assays performed on batches A through E whose results by the U.S.P. XVI procedure are shown in Table I were repeated using the new modified procedure. Aliquots of tablet powder from the same crushed tablets were used. Table VIII shows the results obtained by the two analysts on the five batches of tablets assayed on 5 consecutive days. The poor reproducibility previously noted on the standards and the day to day variation have practically disappeared.

Precision.—A comparison of the precision of the analyses between the U.S.P. XVI method (Table I) and the modified method (Table VIII) using the variances generated by the replicates indicates that the modified method is significantly better than the U.S.P. XVI method as indicated below.

Precision, calculated as σ (from replicates) $\times$ 100/(mean absorbance)

Sample	Method	Precision, %
Standards	U.S.P. XVI	2.3
	Modified	0.8
Tablets	U.S.P. XVI	1.7
	Modified	0.6

Applicability to Other Steroids.—Table IX indicates the rate of color development for steroids having modified environment in the side chain.

TABLE IX.—RATE OF COLOR DEVELOPMENT FOR VARIOUS STEROIDS[a]

	Molar Absorbance		
Steroid	1 Hour	2 Hours	3 Hours
Prednisone	16,600	16,900	16,800
Prednisolone	16,850	16,800	16,900
9α-Fluoro-16α-methyl prednisolone	17,150	17,450	17,100
9α-Fluoro-16β-methyl prednisolone	11,475	15,250	16,450
Desoxycorticosterone	16,500	16,300	16,200
Prednisone-21-acetate	17,000	17,300	17,400
Prednisone-21-hemisuccinate	13,100	15,100	15,150
9α-Fluoro-16α-methyl prednisolone phosphate	0	0	0
9α-Fluoro-16α-methyl prednisolone sulfate	0	0	0

[a] 30° water bath, U.S.P. 95% ethanol, and 1:20 base dilution used.

The color development fails completely when the 21-hydroxyl is firmly bound as in a phosphate or sulfate ester.

SUMMARY

The procedure reported by Johnson, King, and Vickers for bulk hydrocortisone has been extended to the assay of prednisone and prednisolone in tablets. The conditions found necessary to obtain satisfactory reproducibility are use of low actinic glassware, complete evaporation of the chloroform extracts under nitrogen, use of smaller tetramethylammonium hydroxide concentration and its addition last, flushing the empty part of the flasks containing the reagent solution with nitrogen, development of the color in a 30° water bath, and the use of ethanol with controlled water content such as U.S.P. 95% ethanol. When these conditions are fulfilled, the color shows no deterioration even after 2 hours at 30° in a closed flask.

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Application of $\Delta\epsilon$-Analysis to Pharmaceuticals II

Determination of Morphine

By JOSEPH L. CASINELLI and JOSEPH E. SINSHEIMER†

Morphine exhibits a bathochromic displacement typical for phenols of its U.V. absorption spectrum in alkaline solution as compared to its spectrum in acid solution. Subtracting the absorbances of the U.V. spectrum in acid solution from those of the U.V. spectrum in alkaline solution results in a characteristic $\Delta\epsilon$-spectrum (differential spectrum). This difference spectrum exhibits a maximum at 298 mμ of sufficient magnitude to be suitable for quantitative determinations in a manner analogous to the use of the usual U.V. absorption spectra but with added specificity. The method has been applied to the determination of morphine in tablets, tincture of opium, and camphorated tincture of opium.

THE application of $\Delta\epsilon$-analysis (differential analysis) for the determination of eugenol in pharmaceuticals has been described in a previous paper from these laboratories (1). Williams (2, 3) has suggested a similar procedure for the analysis of barbiturates and salicylates. It is the purpose of this investigation to use $\Delta\epsilon$-analysis for the assay of morphine in some representative dosage forms.

Morphine exhibits a bathochromic displacement, typical for phenols, of its U.V. absorption spectrum in alkaline solution as compared to its spectrum in acid solution (4, 5). Subtracting the absorbances of the U.V. spectrum in acid solution from those of the U.V. spectrum in alkaline solution results in a characteristic $\Delta\epsilon$-spectrum (6). This difference spectrum exhibits maxima of sufficient magnitude to be suitable for quantitative determinations in a manner analogous to the use of the usual U.V. absorption spectra.

The difference in absorbance of a sample of morphine was determined at a difference maximum and this value served as the basis for the determination of morphine in tablets, tincture of opium, and camphorated tincture of opium.

EXPERIMENTAL

Ultraviolet absorption measurements were made with a Beckman, model DU spectrophotometer. A Beckman Zeromatic meter was used to determine pH.

Materials.—Morphine monohydrate (Mallinckrodt) was purified by chromatography (7) followed by two recrystallizations from alcohol-water. All reagents were of C.P. grade and were used without additional purification.

Received April 28, 1961, from the Colleges of Pharmacy, University of Rhode Island, Kingston, and University of Michigan, Ann Arbor.

Accepted for publication July 18, 1961.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

Following the acceptance of this paper, a similar procedure has been described by Milos, C., THIS JOURNAL, **50**, 837 (1961).

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Determination of Morphine Spectra.—Samples of about 80 mg. of morphine, accurately weighed, were dissolved in alcohol in 100-ml. volumetric flasks. Ten-milliliter aliquots were transferred to a second set of 100-ml. volumetric flasks and diluted to volume with a series of solutions prepared from standard solutions of sodium hydroxide and sulfuric acid. In this manner the U.V. spectra for 12 solutions of morphine ranging in pH from 3.0 to 12.0 were determined.

Determination of $\Delta\epsilon$ for Morphine.—Samples of about 80 mg. of morphine were accurately weighed, transferred to a 100-ml. volumetric flask, and dissolved by adding alcohol to volume. One 10-ml. aliquot of this solution was diluted to volume with water and 1.00 ml. of 1.000 *N* sodium hydroxide in a 100-ml. volumetric flask. A second 10-ml. aliquot of the alcoholic morphine solution was transferred to a 100-ml. volumetric flask and diluted to volume with water and 1.00 ml. of 0.100 *N* sulfuric acid. The absorbance of the alkaline solution was determined at 298 mμ relative to the acid solution in the reference cell. Blank solutions were run under the same conditions in order to correct for any contribution due to solvents and reagents. A total of 18 determinations were made in this manner. The alkaline solutions were recorded with a pH of 12.0 $\pm$0.1 and the acid solutions with a pH of 3.0 $\pm$0.1.

Assay of Morphine Sulfate Tablets.—Twenty 1/4 gr. tablets of morphine sulfate were weighed and powdered. A portion of this powder, about 90 mg., was accurately weighed, transferred to a 100-ml. volumetric flask, and dissolved by adding water to volume. Two 10-ml. aliquots of this solution were diluted, buffered, and the relative absorbance at 298 mμ was determined as described under the determination of $\Delta\epsilon$ for morphine.

Assay of Tincture of Opium.—A 5-ml. portion of the tincture was transferred to a beaker by pipet, mixed with 5 ml. of water, and reconcentrated on a steam bath to about 5 ml. To insure the removal of alcohol, a second 5-ml. portion of water was added and the mixture reconcentrated to about 5 ml. After cooling, 0.5 ml. of ammonia solution was mixed with the opium extract followed by 2 Gm. of Celite 545. This mixture was quantitatively transferred to the top of a column containing 5 Gm. of Celite 545. A total of 150 ml. of benzene was used to complete the transfer of the Celite-opium residue to the column and to develop the chromatogram. The

elution with benzene was followed by a mixture of chloroform-absolute ethanol (3:1). The material eluted before this change in solvents was discarded, while the eluate after the change was collected to volume in a 100-ml. volumetric flask. A 10-ml. aliquot of this eluate was transferred to a 100-ml. volumetric flask, diluted with 50 ml. of alcohol, 0.50 ml. of 1.000 *N* sodium hydroxide, and water to volume. In a like manner a second 10-ml. aliquot was diluted with 50 ml. of alcohol, 2.00 ml. of 0.100 *N* sulfuric acid, and water to volume in a 100-ml. flask. The differences in absorbances of these two solutions and a set of blank solutions were determined at 298 mμ.

Assay of Camphorated Tincture of Opium.—A 100-ml. portion of camphorated tincture of opium was evaporated to constant volume with the aid of a steam bath and a jet of air. The residue was mixed with 10 ml. of water, reconcentrated to constant volume, treated with ammonia, and chromatographed as described under the assay of tincture of opium. However, 250 ml. of the chloroform-alcohol mixture was required for the complete elution of the morphine. Therefore, a 25-ml. aliquot of this eluate was then transferred to a 100-ml. volumetric flask and brought to volume with alcohol and 0.50 ml. of 1.000 *N* sodium hydroxide. A second 25-ml. aliquot was diluted to volume with alcohol and 2.00 ml. of 0.100 *N* sulfuric acid in a 100-ml. flask. The differences in absorbances of these two solutions and a set of blank solutions were again determined at 298 mμ.

Table I.—Analysis of Morphine Sulfate Pentahydrate in Tablets

	Labeled Concentration	16.2 Gm./Tab.
Found	1	16.3
	2	16.3
	3	16.5
	4	16.2
	5	16.3
	6	16.1
	7	16.9
	8	16.9
	9	17.0
	10	17.0
Av.		16.6

Table II.—Analysis of Anhydrous Morphine in Opium Tinctures

Theoretical Amount		Tincture of Opium 1.00 Gm./100 ml.	Camphorated Opium Tincture 0.0432 Gm./100 ml.
Found	1	1.09	0.0448
	2	1.08	0.0468
	3	1.05	0.0451
	4	1.05	0.0462
	5	1.08	0.0477
	6	1.10	0.0441
	7	1.08	...
	8	1.09	...
	9	1.08	...
	10	1.09	...
Av.		1.08	0.0458

RESULTS AND DISCUSSION

A Δ-absorbance curve for morphine, that is the curve obtained by subtracting the absorbances of the U.V. spectrum in acid solution from those of the U.V. spectrum in alkaline solution, shows maxima at 256 and 298 mμ. The maxima at 298 mμ, even though it is of lower intensity, was utilized because of its stability over larger pH ranges. The difference at 298 mμ was found to be constant provided that the acid solution was at a pH of 5.0 or less and the basic solution was greater than 11.4. To serve as the basis for quantitative determinations of morphine solutions, the difference in molar absorptivity, $\Delta\epsilon$, at this maximum was determined for solutions of known concentrations of anhydrous morphine. The average of 18 determinations resulted in a value of 2,346 for $\Delta\epsilon$, or the difference in absorbance at 298 mμ for molar solutions of morphine at a pH of 12 ±0.1 as compared to molar solutions at a pH of 3.0 ±0.1.

Based upon this value for $\Delta\epsilon$, the assay of various dosage forms of morphine was undertaken. The assay of morphine sulfate in tablets involves the solution of the tablets and the selection of aliquots of this solution so that the difference in absorbance, ΔA, in a basic solution as compared to an acid solution is in the order of 0.3. The amount of morphine sulfate pentahydrate in the aliquot is then calculated by the formula: X Gm./100 ml. = $379.4\ \Delta A/(10\ \Delta\epsilon)$. Table I contains a summary of the results of this analysis of morphine sulfate pentahydrate in 1/4 gr. tablets.

The assay of opium preparations for their morphine content by this procedure still requires preliminary separation. For example, the opium alkaloids narceine, narcotine, and papaverine, as well as some of nonalkaloidal constituents of opium, exhibit differences in U.V. absorption in acid and basic media at 298 mμ.

Grosfeld-Nir, *et al.* (7), have described a chromatographic separation for the determination of morphine in opium. While this procedure is efficient for the separation of morphine from the other opium alkaloids, its application to opium depends upon the careful control of the original extraction of the drug. Under such controlled conditions it is possible to assay for morphine by alkalimetry following chromatographic separation. Tincture of opium or camphorated tincture of opium assayed by this procedure did not give quantitative results for morphine. However, the chromatographic procedure in combination with $\Delta\epsilon$-analysis did give satisfactory results.

Table II is a summary of the results of the analysis of these tinctures by chromatography followed by $\Delta\epsilon$-analysis. The labeled concentration of the commercial tincture of opium was 1 Gm. per 100 ml. The camphorated opium tincture was prepared from the tincture of opium. On the basis of our analysis of 1.08 Gm. of morphine per 100 ml. for this tincture of opium, the camphorated tincture was calculated to contain 0.0432 Gm. of morphine per liter.

CONCLUSION

It is concluded from the results of this investigation that $\Delta\epsilon$-analysis has value for the determi-

nation of morphine in dosage forms and has the advantage of greater specificity as compared to direct U.V. measurements.

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Evaluation and Mechanisms of Action of Several Experimental Hypotensive Agents

By WILLIAM J. HUDAK†, WILLIAM J. KINNARD, Jr., and JOSEPH P. BUCKLEY

The hypotensive activity of [1-methyl-3-(2'5'6'-trimethyl-1'-cyclohexenyl)propyl] [3-morpholinopropyl] dimethyl ammonium bromide methobromide (RO 2-7832), (+) - 6 - methyl - 9 - diethylaminomethyl - 10 - hydroxy - 1,2,3,3a,5,6,6a,7,11b,11c-decahydro-4H-dibenzo [de,g] quinoline dihydrobromide (RO 2-9618), and 8,8'-bis(2-dimethylaminoethoxy)-6,6'-bithiachroman-4,4'-dione dihydrochloride (RO 2-9811) were investigated in anesthetized rats and dogs. Central and peripheral hypotensive activities were studied utilizing dog cross circulation preparations; and included trimethidinium methosulfate and beta-dimethyl-aminoethyl-N-methylpipecolinate dimethobromide (JB-591) as well as the previously mentioned compounds. RO 2-7832 and trimethidinium appeared to act as potent ganglionic blockers. The hypotensive activity of RO 2-9618 was due to both ganglionic blockade and mild adrenolytic activity. JB-591 was the only compound that demonstrated any central hypotensive activity.

ALTHOUGH the etiology of essential hypertension is still not fully understood, many investigators agree that the reduction in arterial blood pressure is beneficial to the hypertensive patient and will, in many instances, prolong life (1–4). Even though there are many hypotensive compounds available, there is still a need for safer therapeutic agents possessing a minimum of side effects, thereby permitting the patient to lead a useful, productive life. The mechanism of action of the compound should be understood so that the drugs can be intelligently used by the physician in the control of hypertensive cardiovascular disease. This present report is mainly concerned with the evaluation of the hypotensive activity of RO 2-7832,[1] RO 2-9618,[1] and RO 2-9811[1] (Fig. 1), and the mechansim of action of these compounds, and trimethidinium[2] (5) and JB-591[3] (6–8).

Received April 28, 1961, from the Cardiovascular Research Laboratories, Department of Pharmacology, University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.
Accepted for publication June 8, 1961.
This investigation was supported in part by research grant No. H-3475, National Heart Institute and a grant from Hoffmann-LaRoche, Inc., Nutley, N. J.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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[1] Kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.
[2] Kindly supplied as WY-1395 (Ostensin) by Wyeth Laboratories, Philadelphia, Pa.
[3] Kindly supplied by Lakeside Laboratories, Inc., Milwaukee, Wis., (beta-dimethylaminoethyl-N-methylpipecolinate dimethobromide).

EXPERIMENTAL

Hypotensive Activity in Rats.—The RO compounds were screened and evaluated in anesthetized Wistar rats (urethan, 1.25 Gm./Kg., i.p.) utilizing the method described in a previous paper (9). Direct blood pressure was recorded via the left common carotid artery onto a slowly moving kymograph. The compounds were dissolved prior to use in distilled water and administered via a femoral vein. Hexamethonium, 5 mg./Kg., was utilized as a control compound in this phase of the study.

The oral activity of the three RO compounds was investigated in: (*a*) anesthetized normotensive albino rats, (*b*) unanesthetized normotensive rats, and (*c*) unanesthetized hypertensive rats.

The animals in (*a*) were fasted for 24 hours prior to their use. They were anesthetized with urethan, 1.25 Gm./Kg., i.p., and prepared for direct blood pressure recording. The proximal end of the trachea was cannulated with polyethylene tubing (2 mm. o.d. and 1.5 mm. i.d.). Fresh solutions of the compounds were administered via gastric intubation utilizing a Davol No. 8 French rubber catheter, and each dose was diluted with distilled water to a volume of 2 ml.

The blood pressures of unanesthetized normotensive rats (*b*) were obtained utilizing the photoelectric tensometer (10). Albino Wistar rats were trained for a period of one week prior to the initiation of the study. The animals were fasted for 24 hours, after which fresh solutions of the compounds were administered via gastric intubation as previously described. Blood pressures were obtained periodically prior to and after drug administration.

nation of morphine in dosage forms and has the advantage of greater specificity as compared to direct U.V. measurements.

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Evaluation and Mechanisms of Action of Several Experimental Hypotensive Agents

By WILLIAM J. HUDAK†, WILLIAM J. KINNARD, Jr., and JOSEPH P. BUCKLEY

The hypotensive activity of [1-methyl-3-(2'5'6'-trimethyl-1'-cyclohexenyl)propyl] [3-morpholinopropyl] dimethyl ammonium bromide methobromide (RO 2-7832), (+) - 6 - methyl - 9 - diethylaminomethyl - 10 - hydroxy - 1,2,3,3a,5,6,6a,7,11b,11c-decahydro-4H-dibenzo [de,g] quinoline dihydrobromide (RO 2-9618), and 8,8'-bis(2-dimethylaminoethoxy)-6,6'-bithiachroman-4,4'-dione dihydrochloride (RO 2-9811) were investigated in anesthetized rats and dogs. Central and peripheral hypotensive activities were studied utilizing dog cross circulation preparations; and included trimethidinium methosulfate and beta-dimethyl-aminoethyl-N-methylpipecolinate dimethobromide (JB-591) as well as the previously mentioned compounds. RO 2-7832 and trimethidinium appeared to act as potent ganglionic blockers. The hypotensive activity of RO 2-9618 was due to both ganglionic blockade and mild adrenolytic activity. JB-591 was the only compound that demonstrated any central hypotensive activity.

ALTHOUGH the etiology of essential hypertension is still not fully understood, many investigators agree that the reduction in arterial blood pressure is beneficial to the hypertensive patient and will, in many instances, prolong life (1–4). Even though there are many hypotensive compounds available, there is still a need for safer therapeutic agents possessing a minimum of side effects, thereby permitting the patient to lead a useful, productive life. The mechanism of action of the compound should be understood so that the drugs can be intelligently used by the physician in the control of hypertensive cardiovascular disease. This present report is mainly concerned with the evaluation of the hypotensive activity of RO 2-7832,[1] RO 2-9618,[1] and RO 2-9811[1] (Fig. 1), and the mechansim of action of these compounds, and trimethidinium[2] (5) and JB-591[3] (6–8).

Received April 28, 1961, from the Cardiovascular Research Laboratories, Department of Pharmacology, University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.
Accepted for publication June 8, 1961.
This investigation was supported in part by research grant No. H-3475, National Heart Institute and a grant from Hoffmann-LaRoche, Inc., Nutley, N. J.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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[1] Kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.
[2] Kindly supplied as WY-1395 (Ostensin) by Wyeth Laboratories, Philadelphia, Pa.
[3] Kindly supplied by Lakeside Laboratories, Inc., Milwaukee, Wis., (beta-dimethylaminoethyl-N-methylpipecolinate dimethobromide).

EXPERIMENTAL

Hypotensive Activity in Rats.—The RO compounds were screened and evaluated in anesthetized Wistar rats (urethan, 1.25 Gm./Kg., i.p.) utilizing the method described in a previous paper (9). Direct blood pressure was recorded via the left common carotid artery onto a slowly moving kymograph. The compounds were dissolved prior to use in distilled water and administered via a femoral vein. Hexamethonium, 5 mg./Kg., was utilized as a control compound in this phase of the study.

The oral activity of the three RO compounds was investigated in: (*a*) anesthetized normotensive albino rats, (*b*) unanesthetized normotensive rats, and (*c*) unanesthetized hypertensive rats.

The animals in (*a*) were fasted for 24 hours prior to their use. They were anesthetized with urethan, 1.25 Gm./Kg., i.p., and prepared for direct blood pressure recording. The proximal end of the trachea was cannulated with polyethylene tubing (2 mm. o.d. and 1.5 mm. i.d.). Fresh solutions of the compounds were administered via gastric intubation utilizing a Davol No. 8 French rubber catheter, and each dose was diluted with distilled water to a volume of 2 ml.

The blood pressures of unanesthetized normotensive rats (*b*) were obtained utilizing the photoelectric tensometer (10). Albino Wistar rats were trained for a period of one week prior to the initiation of the study. The animals were fasted for 24 hours, after which fresh solutions of the compounds were administered via gastric intubation as previously described. Blood pressures were obtained periodically prior to and after drug administration.

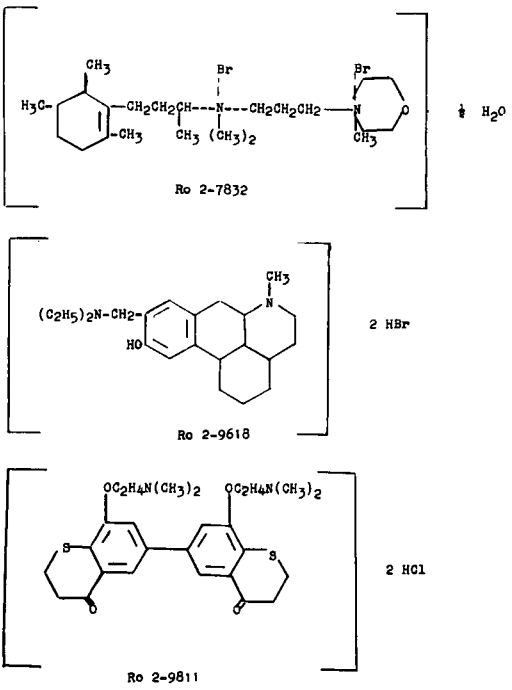


Fig. 1.—Structures of the compounds evaluated for hypotensive activity.

Hypertensive rats (*c*) were prepared by applying a figure-of-eight ligature to the right decapsulated kidney and removing the contralateral kidney 1 week later. After an additional 4-week period, the rats received 6.25 mg. of desoxycorticosterone trimethylacetate,[4] intramuscularly, and 0.9% saline drinking solution once weekly. Of 48 rats, 40 developed arterial pressures in excess of 180 mm. Hg 8 weeks after the initial operation. Fresh solutions of the compounds were administered orally to rats fasted for 24 hours and the blood pressures determined, utilizing the photoelectric tensometer, prior to and periodically after administering the compounds.

Ganglionic Blocking Activity.—Adult cats of both sexes were anesthetized with an intraperitoneal injection of pentobarbital sodium, 35 mg./Kg. Blood pressure was recorded from the right femoral artery via a mercury manometer. The preganglionic segment of the cervical sympathetic nerve was stimulated with a submaximal stimulus (determined for each animal) using a Harvard stimulator at a frequency of 60 impulses per second for 10 seconds, and the effect on the nictitating membrane recorded. Three such stimuli were applied at 5-minute intervals and the average response determined. The submaximal stimulus was also determined for the postganglionic fiber. The responses obtained after injection of the test compound were reported as per cent of the mean response. The antagonism of the experimental compounds to the hypertensive activity of 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) (11–13) were also studied in the same preparations. The mean pressor response to several 10 mcg./Kg. intravenous injections of DMPP prior to administering the experimental compounds was obtained, and the degree of inhibition of the pressor response after administration of the compounds was calculated in terms of per cent of the mean control response. The effects of the compounds in antagonizing the pressor response obtained by bilateral carotid occlusion were also determined in a similar manner.

Hypotensive Activity in Dogs.—The hypotensive activities of the compounds were studied in unanesthetized normotensive dogs. A permanent catheter (Bardic 10) was inserted into a femoral artery permitting direct recording of the blood pressure onto a slowly moving kymograph by way of a mercury manometer (14). Each animal received a particular compound via the oral or intravenous routes to determine the approximate degree of oral absorption. The dogs were starved 24 hours prior to oral administration, and the oral dose equivalent to 10 times the i.v. dose was administered in capsule form. A minimum of two days elapsed prior to administering the compounds via the intravenous route. Approximately one week after receiving the last dose of the compound, the animal was anesthetized with pentobarbital sodium, 35 mg./Kg., i.v., and the blood pressure recorded via the femoral catheter onto a slowly moving kymograph. The trachea was cannulated and the common carotid arteries isolated in the usual manner. The vagi were isolated, doubly ligated, and the nerves sectioned between the ligatures. The following tests were utilized at 5-minute intervals: (*a*) the stimulation of the central vagal stump (35 volts at a frequency of 60 per second for 10 seconds); (*b*) stimulation of the caudal vagal stump utilizing the same stimuli as described above; (*c*) bilateral carotid occlusion for 10 seconds; (*d*) DMPP (10 mcg./Kg.) i.v.; (*e*) epinephrine (1 ml. of 1:100,000) i.v.; (*f*) histamine base (2.5 mcg./Kg.) i.v. The mean per cents of two responses prior to medication were utilized as controls and the degree of augmentation or inhibition was calculated in terms of the per cent of this mean control response.

Dog Cross Circulation Studies.—The following procedure was utilized to produce a vascularly isolated, neurally intact head preparation: The recipient dog was anesthetized with pentobarbital sodium, 35 mg./Kg., i.v., and the blood pressure recorded via the right femoral artery by way of a mercury manometer onto a slowly moving kymograph. The trachea was cannulated and both common carotid arteries and jugular veins isolated. The internal jugular veins were doubly ligated and sectioned. A circumferential incision was then made around the midneck and the skin retracted. All the neck musculature from C-2 to C-5 was separated and removed using electrocautery. A dorsal laminectomy was performed between C-2 and C-3 or C-3 and C-4 to expose the underlying cord. The cord was gently retracted and a soft rubber sponge (0.75 cm. × 4.0 cm. × 0.25 cm.), through which a length of 21-gauge steel wire had been passed, was inserted between the spinal cord and dorsal surface of the centrum. The wire was looped around the central portion of the spinal column and guided into the intervertebral space and passed between the two common carotid arteries. The wire was then tightened with a Shifrin wire tightener

[4] Generously supplied as Percorten by Ciba Pharmaceutical Products, Inc., Summit, N. J.

to occlude the venous sinuses and vertebral arteries and the tightener left in place. The dog was placed ventral side up and artificial respiration administered. The left rib cage was exposed and the pleural cavity opened between the third and fourth ribs, utilizing a chest spreader. Loose ligatures were placed around the innominate and left subclavian arteries and superior vena cava (caudal to the entry of the vertebral veins). The donor dog (approximately 25% larger than the recipient) was anesthetized and the blood pressure recorded via the right femoral artery. The donor was heparinized with 2,000 to 4,000 units of heparin intravenously, and 50 mg. of pyribenzamine was administered subcutaneously to prevent possible anaphylactic reactions. Anastomosis between the recipient's two common carotid arteries and two external jugular veins with the corresponding left vessels of the donor was accomplished, and the ligatures around the innominate and left subclavian arteries and superior vena cava tightened securely (see Fig. 2). After both donor and recipient blood pressures had stabilized, 0.25 ml. of Lugol's solution (U.S.P.) was injected into the tubing supplying the blood to the head of the recipient. Approximately 15 minutes later, 10 μc. of iodine^{131} (radio-iodinated serum albumin, human, Squibb) was administered intra-arterially into the head of the recipient. Circulatory leakage between the head and body of the recipient animal was determined at 5, 15, 30, 60 minutes, and then at hourly intervals after administration of radioactive iodine, using the procedure described in a previous paper (15). The mean leakage in 19 experiments, at the end of 3 hours, was 3.1%. The compounds were administered via the arterial inflow to the recipient's head.

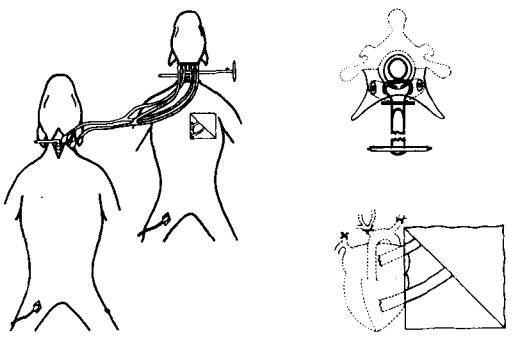


Fig. 2.—Diagrammatic illustration of the cross circulation technique used.

RESULTS

Hypotensive Activity in Rats.—The hypotensive activity of the RO compounds, administered intravenously, is summarized in Table I. RO 2-7832 appeared to be the most active hypotensive compound investigated as 1 mg./Kg., i.v., produced marked hypotensive effects persisting for over 4 hours. The compound was also very active when administered orally to anesthetized normotensive rats. RO 2-7832, in doses ranging between 1 and 20 mg./Kg., produced drops in blood pressure between 40 and 55%, the onset of action occurring between 15 and 31 minutes, and the hypotensive effects persisting for approximately 8 hours after the onset of action. RO 2-7832 was also the most active compound when administered orally to unanesthetized normotensive albino rats. The onset of action usually occurred within 60 minutes, and the duration of action was usually over 8 hours. A decrease in oral and nasal secretions and marked pupil dilatation were observed with the onset of the hypotensive effects. RO 2-7832 was administered to 10 hypertensive rats. Doses of 20 mg./Kg. produced a mean drop in blood pressure of 26% and the maximum hypotensive effect occurred approximately 100 minutes after the administration of the compound (see Tables II and III). The average duration of action was in excess of 354 minutes; however, the blood pressures of four of the rats had not returned to predrug levels after 8 hours. Dryness of the mouth and marked pupil dilatation were observed with the onset of drug action.

Ganglionic Blocking Activity.—The ganglionic blocking activity and the effect on bilateral carotid occlusion pressor response of the RO compounds on normotensive cats are summarized in Table IV. RO 2-7832 elicited powerful ganglionic blockade and almost completely blocked the pressor response to DMPP. Moderate ganglionic blocking activity was produced by RO 2-9618, and RO 2-9811 had little effect on ganglionic transmission. All of the compounds inhibited the pressor response to bilateral carotid occlusion with RO 2-7832 demonstrating the least activity in inhibiting this pressor reaction.

Hypotensive Activity in Dogs.—The effects of the RO compounds on the blood pressure of unanesthetized normotensive dogs are summarized in Table V. RO 2-7832 appeared to be the most active compound when administered either intravenously or orally. The compound produced almost complete relaxation of the nictitating membrane, dryness of the eyes and mouth, mydriasis, and loss of pupillary reflex. The effects of the RO compounds on the blood pressure of anesthetized normotensive dogs and the physiological responses to electrical stimulation of the central vagal stump and caudal vagal stump, bilateral carotid occlusion, DMPP, epinephrine, and histamine are summarized in Table VI. The pressor response to central vagal stump stimulation was completely inhibited in one instance by 2 mg./Kg. of RO 2-7832. RO 2-9811 had no apparent effects upon this physiological response. The depressor response to stimulation of the caudal vagal stump was completely blocked by RO 2-7832 in one dog, and a pressor response was obtained in another. The other compounds decreased the depressor response. The pressor response produced by bilateral carotid occlusion was completely inhibited by RO 2-7832, RO 2-9618 had little or no effect, and RO 2-9811 inhibited this response by 40 and 68%. The pressor response to DMPP, i.v., was blocked by RO 2-7832 and augmented by RO 2-9811 and RO 2-9618. The pressor response to exogenous epinephrine was enhanced by RO 2-7832 and RO 2-9811 and slightly diminished by RO 2-9618.

Cross Circulation Studies.—The effects of the compounds on the dog cross circulation preparations are summarized in Table VII. JB-591 was the only compound which appeared to produce a centrally mediated hypotensive effect (Fig. 3). Marked de-

TABLE I.—EVALUATION OF CERTAIN EXPERIMENTAL COMPOUNDS ON THE BLOOD PRESSURE OF NORMOTENSIVE RATS[a]

Compound	Dose, mg./Kg.	No. of Rats	Drop in Blood Pressure, % ± S.D.	Mean Time to Return to Original Level, min. ± S.D.	Rating[b]
RO 2-7832	1.0	6	44.8 ± 4.1	264 ± 35.9	++++
RO 2-9811	10.0	5	35.2 ± 7.8	52 ± 24.1	++
RO 2-9618	25.0	6	46.0 ± 5.9	6 ± 2.8	+
Hexamethonium	5.0	8	46.5 ± 17.9	30 ± 15.2	+

[a] Administered intravenously, rats anesthetized with urethan, 1.25 Gm./Kg., i.p. [b] Rating +, 0–40 min. duration; ++, 41–80 min.; +++, 81–120 min.; ++++, 121+ min.

TABLE II.—EFFECTS OF 20 MG./KG., RO 2-7832, *Per Os*, ON THE BLOOD PRESSURE OF UNANESTHETIZED HYPERTENSIVE RAT PREPARATIONS[a]

Weight, Gm.	245	215	208	241	281	176	135	138	147	148
Time, min.	Blood Pressure, mm. Hg									
0	204	232	236	214	196	198	198	204	204	218
15	...	215	230	188	214	168	178	212	...	212
30	136	...	202	170	178	164	180	182	188	230
60	140	164	...	...	...	...	...	...	156	182
65	...	...	198	162	164	174	154	176	...	...
90	...	...	182	158	166	...	...	...	...	194
120	152	182	176	162	...	...	...	...	164	...
125	...	...	...	...	178	178	172	170	...	...
160	...	186	194	184	188	...	...	...	...	172
180	152	...	...	...	...	192	...	...	144	...
185	...	...	...	188	192	...	196	184	...	...
240	166	220	210	212	206	208	194	192	120	148
300	192	220	214	204	200	204	202	190	144	192
360	222	214	214	210	192	...	...	...	146	160
420	206	214	210	...	...	...	...	...	142	166
480	206	218	210	...	...	...	...	...	146	158
Maximum % drop	33.3	29.3	25.4	26.1	16.3	17.1	22.2	16.6	41.1	32.1
Mean maximum % drop	26.0 ± 8.2									

[a] S.D. = $\sqrt{\frac{\epsilon(x-\bar{x})^2}{n-1}}$.

TABLE III.—SUMMARIZATION OF THE EFFECTS OF CERTAIN HYPOTENSIVE COMPOUNDS (*Per Os*) ON THE BLOOD PRESSURE OF UNANESTHETIZED HYPERTENSIVE RAT PREPARATIONS

Compound	No. of Rats	Dose, mg./Kg.	Mean Onset of Hypotensive Activity, min.	Mean Time of Maximum Hypotensive Activity, min.	Mean Maximum % Drop	Mean Duration of Action, min.
RO 2-7832	10	20	21	106.5	26.0	354+
RO 2-9811	6	50	20	65.3	23.4	247
RO 2-9618	6	50	15	47.5	20.9	230

TABLE IV.—SUMMARIZATION OF THE GANGLIONIC BLOCKING ACTIVITY AND THE EFFECT ON THE BILATERAL CAROTID OCCLUSION PRESSOR RESPONSE OF CERTAIN HYPOTENSIVE AGENTS ON NORMOTENSIVE CATS

Compound	Animal No.	Dose, mg./Kg.	Drop in Blood Pressure, %	Duration,[a] min.	Sympathetic Ganglionic Blockade, %	DMPP Blockade, %	Bilateral Carotid Occlusion Blockade, %
RO 2-7832	1	0.5	77.4	55	100	100	30.7
	2	0.5	61.6	168	100	75	0.0
	3	0.5	68.2	297	100	85.7	84.2
RO 2-9811	1	5.0	53.4	30	0	0	69.6
	2	5.0	50.5	98	12.6	0	75.0
	3	5.0	55.3	96	0	23.5	53.5
RO 2-9618	1	15.0	60.0	57	32.8	0	86.0
	2	15.0	60.0	83	15.8	43.2	73.3
	3	15.0	62.5	61	32.0	0	53.3

[a] Experiments terminated, all blood pressures below original levels.

TABLE V.—EFFECT OF THE EXPERIMENTAL COMPOUNDS, *Per Os* AND *I.V.*, ON THE BLOOD PRESSURE OF UNANESTHETIZED NORMOTENSIVE DOGS

Dog No.	Weight, Kg.	Sex	Compound	Dose, mg./Kg.	Original Blood Pressure, mm. Hg	Onset of Drug Action, min.	Time of Maximum Drop, min.	Maximum Drop, mm. Hg	Maximum % Drop	Time to Return to Original Level, min.
1	7.04	F	RO 2-7832	20 (*per os*)	122	8	38	52	42.6	746[a]
	6.61	F	RO 2-7832	2 (*i.v.*)	120	immediate	3	40	33.3	396[b]
2	12.0	F	RO 2-7832	20 (*per os*)	146	15	22	16	10.9	276
	12.0	F	RO 2-7832	2 (*i.v.*)	156	immediate	2	50	32.0	190[c]
3	7.55	F	RO 2-9811	50 (*per os*)	100	.....	..	0	0	..[d]
	7.30	F	RO 2-9811	5 (*i.v.*)	100	immediate	1	60	60	75
4	7.55	F	RO 2-9811	50 (*per os*)	96	64	73	16	16.6	50
	7.48	F	RO 2-9811	5 (*i.v.*)	100	immediate	1	60	60	53
5	7.6	M	RO 2-9618	150 (*per os*)	130	26	84	50	38.4	114
	6.55	M	RO 2-9618	15 (*i.v.*)	124	immediate	2	56	45.1	92
6	7.0	M	RO 2-9618	150 (*per os*)	104	15	22	34	32.6	64
	7.5	M	RO 2-9618	15 (*i.v.*)	108	.....	..	..	..	126[e]

[a] Experiment terminated, blood pressure 40 mm. Hg from original level. [b] Experiment terminated, blood pressure 52 mm. Hg from original level. [c] Experiment terminated, blood pressure 34 mm. Hg from original level. [d] No effect in 266 minutes. [e] No effect in 126 minutes.

TABLE VI.—CARDIOVASCULAR ACTION OF THE EXPERIMENTAL COMPOUNDS ON NORMOTENSIVE ANESTHETIZED DOGS

						Per Cent Response of Control Value					
Dog No.	Weight, Kg.	Sex	Compound	Dose, mg./ Kg.	Original Blood Pressure	% Drop	Central Vagal Stump Stimulation	Caudal Vagal Stump Stimulation	Bilateral Carotid Occlusion	DMPP	Epinephrine	Histamine Base
1	6.4	F	RO 2-7832	2	110	45	0	[a]	0	0	317	132
2	11.0	F	RO 2-7832	2	192	43	122	0	0	0	545	95
3	6.78	F	RO 2-9811	5	130	45	100	38	68	126	153	79
4	7.2	F	RO 2-9811	5	150	46	92	21	40	228	143	72
5	6.8	M	RO 2-9618	15	146	41	78	44	84	138	88	97
6	7.3	M	RO 2-9618	15	106	42	280	69	100	103	88	100

[a] Pressor response obtained.

pressor responses were produced in all of the recipient animals by JB-591. In most instances, the maximum hypotensive effect occurred more rapidly in the donor than in the recipient. All of the compounds produced marked hypotensive effects in the donor animals. Neither hexamethonium chloride nor trimethidinium (Fig. 4) produced any effect upon the recipient's blood pressure and it would appear that these compounds induce hypotensive effects purely through their peripheral ganglionic blocking activity.

DISCUSSION

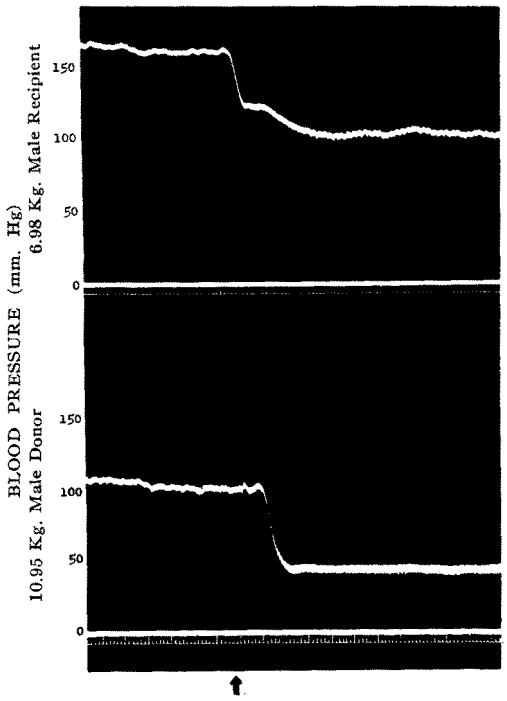
RO 2-7832 appeared to be the most potent of the RO compounds investigated. It produced marked hypotensive effects for prolonged periods of time in normotensive anesthetized rats, dogs, and cats. The cats appeared to be especially sensitive to this compound as minute doses produced marked hypotensive responses. The depressor response, produced by 10 times the intravenous dose of RO 2-7832 administered orally to unanesthetized normotensive and hypertensive rats, was approximately 50% that produced by intravenous administration to anesthetized rats. The depressor response produced by the compound following oral or intravenous administration to unanesthetized dogs was also markedly reduced in contrast to the response in anesthetized animals. This would indicate that the anesthesia had an augmentory action on the hypotensive activity of RO 2-7832. The following physiological responses indicate that RO 2-7832 is a ganglionic blocking agent: (*a*) complete inhibition of the pressor response of DMPP; (*b*) inhibition of the contraction of cat nictitating membrane to preganglionic stimulation of the superior cervical sympathetic nerve (nictitating membrane responded to stimulation of the postganglionic fiber); (*c*) inhibition of the depressor response produced by bilaterial carotid occlusion· (*d*) inhibition of the depressor response produced by caudal vagal stump stimulation; (*e*) marked enhancement of the pressor response to exogenous epinephrine. The compound did not exhibit any evidence of central hypotensive activity (Fig. 5).

Although RO 2-9618 was the least effective of the compounds investigated in the anesthetized rat evaluation studies, it appeared to be one of the most effective compounds when administered via the oral route; however, the duration of action was extremely short. The compound augmented the response produced by DMPP in the dog and suppressed the depressor response produced by stimulation of the caudal vagal stump in the dog. It also partially blocked the pressor response produced by DMPP and contraction of the nictitating membrane to preganglionic stimulation in the cat. Slight adrenergic blocking activity was also indicated by the partial suppression of the pressor response to exogenous epinephrine, and it appears that the hypotensive activity of RO 2-9618 is due to ganglionic blockade and a mild adrenergic blocking action.

TABLE VII.—EFFECT OF THE EXPERIMENTAL COMPOUNDS ON THE DOG CROSS CIRCULATION PREPARATIONS

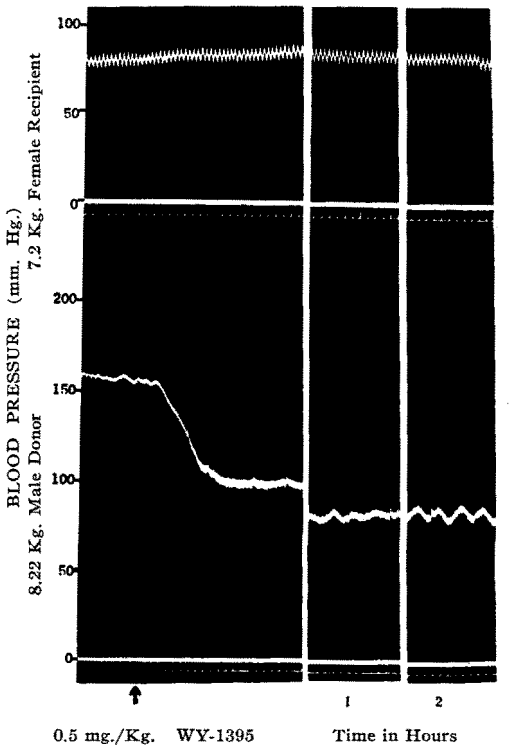
Expt. No.	Compound	Dose,[a] mg./Kg.	Donor Weight, Kg.	Donor Sex	Donor Original Blood Pressure, mm. Hg	Donor Maximum Drop, mm. Hg	Donor Maximum % Drop	Recipient Weight, Kg.	Recipient Sex	Recipient Original Blood Pressure, mm. Hg	Maximum Effect, mm. Hg	Maximum % Effect
1	RO 2-7832	2	10.02	M	112	52	46.4	8.03	F	86	+6	+6.9
2	RO 2-7832	2	11.12	M	134	74	55.2	6.67	F	82	no effect	...
3	RO 2-7832	5	10.72	M	160	118	74.0	7.25	F	78	+10	+13.5
4	RO 2-7832	5	7.86	F	70	30	42.8	7.06	F	74	no effect	...
5	RO 2-7832	5	8.20	M	80	46	57.5	8.99	F	72	−30	−41.7
6	RO 2-9811	5	7.00	F	102	68	66.7	6.69	F	124	+236	+190.0
7	RO 2-9811	5	7.42	F	120	46	38.3	6.48	F	88	no effect	...
8	RO 2-9618	15	7.44	F	114	88	78.2	6.68	M	82	no effect	...
9	RO 2-9618	15	10.39	M	80	50	62.5	7.24	M	126	+10	+7.9
10	JB-591	10	10.35	F	160	90	56.3	7.38	F	80	−50	−62.5
11	JB-591	10	10.95	M	100	58	58.0	6.98	M	158	−100	−63.3
12	JB-591	10	9.95	F	104	60	57.7	6.73	F	100	−54	−54.0
13	JB-591	10	10.20	M	104	42	40.4	6.54	F	114	−38	−33.3
14	Hexameth.[b]	3	6.70	M	88	42	47.7	6.01	F	70	no effect	...
15	Hexameth.	3	6.84	M	86	44	51.2	6.66	F	96	no effect	...
16	WY-1395	0.5	7.85	M	138	92	66.7	6.96	F	72	no effect	...
17	WY-1395	0.5	8.22	M	156	102	65.4	7.26	M	80	no effect	...
18	WY-1395	0.5	10.60	M	108	50	53.7	6.00[c]	F	74	no effect	...
19	WY-1395	0.5	13.70	F	80	40	50.0	6.90[c]	F	76	+10	+13.0

[a] Calculated on donor's weight. [b] Hexamethonium chloride. [c] Bilateral denervation of the carotid sinus-body areas.



10 mg./Kg. JB-591 IA-Recipient

Fig. 3.—The effects of JB-591, 10 mg./Kg., via the recipient carotid inflow, on the peripheral arterial pressures of the recipient and donor dogs.



0.5 mg./Kg. WY-1395 Time in Hours

Fig. 4.—The effects of trimethidinium (WY-1395), 0.5 mg./Kg., via the recipient carotid inflow, on the peripheral arterial pressures of the recipient and donor dogs.

RO 2-9811 produced mild hypotensive effects when administered intravenously and orally to dogs. It also produced a marked depression of respiration. The pressor response caused by bilateral carotid occlusion was inhibited approximately 50%, suggesting possible reflex or ganglionic blocking activity; however, the compound had relatively little effect on ganglionic transmission. It appears that RO 2-9811 may possibly lower blood pressure through some reflex mechanism.

Although trimethidinium has been suggested to have a dual mechanism of action (ganglionic

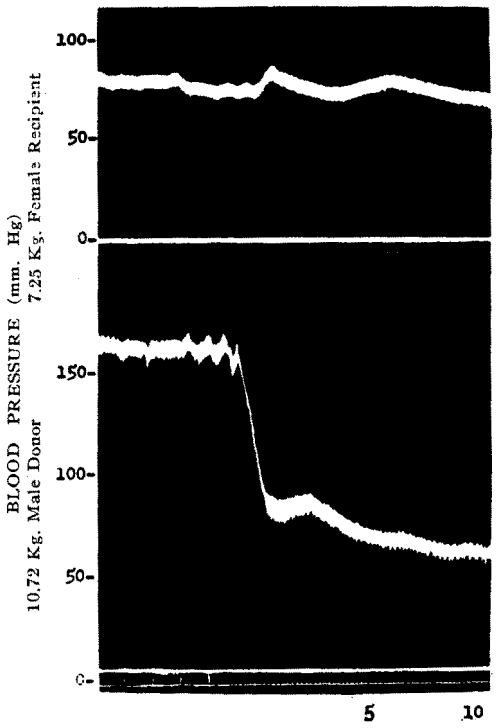


Fig. 5.—The effects of RO 2-7832, 5.0 mg./Kg., via the recipient carotid inflow, on the peripheral arterial pressures of the recipient and donor dogs.

blockade and some form of central depression) (16), the cross circulation studies indicated that the compound does not possess any central hypotensive activity. Since both trimethidinium and hexamethonium have been reported to be potent ganglioplegic agents, it would appear that the hypotensive effects produced by both trimethidinium and hexamethonium are essentially due to their ganglionic blocking activity.

JB-591 was the only compound to demonstrate a centrally mediated hypotensive response. Previous papers have shown the compound to possess ganglionic blocking properties, and it appears that the compound has a dual mechanism of action, namely ganglionic blockade and a central hypotensive action. Clinical studies have shown this compound to possess some tranquilizing action which would further strengthen the possibility of the compound possessing a centrally mediated hypotensive property.

SUMMARY

1. Three new compounds were evaluated for their hypotensive activity in anesthetized normotensive rats.

2. [1 - Methyl - 3 - (2′,5′,6′ - trimethyl - 1′-cyclohexenyl) propyl] [3-morpholinopropyl] dimethyl ammonium bromide methobromide (RO 2-7832) was the most potent hypotensive compound investigated.

3. Central hypotensive activities were studied utilizing dog cross circulation preparations.

4. Trimethidinium and hexamethonium did not elicit central hypotensive responses and apparently act through peripheral ganglionic blockade.

5. JB-591 (β-dimethyl-aminoethyl-N-methyl-pipecolinate dimethobromide) was the only compound to produce a centrally mediated hypotensive response and apparently lowers blood pressure through both ganglionic blockade and some central depressor mechanism.

6. The hypotensive activity of RO 2-7832 was produced predominantly by ganglionic blockade.

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Yohimbine Potentiation of Reserpine Blepharoptosis

By MARVIN H. MALONE and ROBERT H. ROTH, Jr.†

Yohimbine, with an oral LD_{50} in mice of 36.5 mg./Kg. (S.E. = ±3.02), does not show any reserpine-like sedative-blepharoptotic activity in nonlethal dosage. Using factorially designed, graded response assays, yohimbine was shown to potentiate reserpine palpebral ptosis significantly for at least 5 hours when administered concurrently with and at the same oral dosage level as reserpine. Yohimbine administered in higher dosage extended the potentiation effect to a greater degree and for longer periods of time (≥24 hours). Since yohimbine is reported to be both an adrenolytic agent and an antimetabolite of serotonin, this study may indicate that the mechanism of action of reserpine sedation involves depletion of norepinephrine more than serotonin release as a neurohormone. Male mice were more susceptible to reserpine ptosis than females.

YOHIMBINE is a classic adrenolytic agent and recently has been shown to have central nervous system activity (1, 2). Yohimbine is also an antimetabolite of serotonin (3). At the present time there are several theories as to the mechanism of action of reserpine, but all theories implicate two compounds with possible neurohormonal activity in the central nervous system; these compounds are serotonin and norepinephrine (4–6). Since both reserpine and yohimbine have been reported to be natural constituents of *Rauwolfia serpentina* Benth (7, 8), it appeared to be of theoretical and practical interest to determine whether yohimbine would either potentiate or antagonize the action of reserpine. A persistant blepharoptosis is characteristic only of the reserpine-like ataraxics, since the eyelid closure of the phenothiazine ataraxics is easily reversed by environmental stimulation. Rubin, *et al.* (9, 10), have devised a graded response assay for reserpine-like activity utilizing the palpebral ptosis of mice recorded 5 hours after oral dosage. This ptosis is generally considered to be due to central depression and is seen concurrent with facilitation of caffeine and pentylenetetrazol induced extensor seizures; these phenomena are antagonized by prior or simultaneous iproniazid administration (11). Since localized lesions of the human hypothalamus, as in Horner's syndrome, produce reserpine-like symptoms: miosis, enophthalmus, hypothermia, lethargy, and palpebral ptosis (12), it was decided to utilize the mouse ptosis assay to determine the interactions of reserpine and yohimbine.

Received April 28, 1961, from the Division of Pharmacology and the Pharmacy Research Institute, University of Connecticut, Storrs.

Accepted for publication June 6, 1961.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April, 1961.

This project was supported in part by a Lederle Pharmacy Faculty Award.

This paper is dedicated to Dr. Harald G. O. Holck on the occasion of his retirement from the Institute for Cellular Research and the Department of Pharmacology of the University of Nebraska.

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EXPERIMENTAL

General.—White mice were obtained from Joseph E. Stocker, Ramsey, N. J., and were maintained in this laboratory on Purina Laboratory Chow and water *ad libitum* for at least 4 days prior to test. Only mice within a weight range of 15–25 Gm. were used for the ptotic assays. Animals were taken off food 3 to 4 hours prior to dosing and placed back on food 10 hours after dosing. Water was allowed throughout the entire test period. A dosage volume of 0.6 ml./20 Gm. of mouse was constant throughout this study. Animals were dosed utilizing precision grade syringes and cut-off, blunted, polished, 30-mm. long, 20 gauge hypodermic needles. Test solutions were coded using randomized numbers, so that the two dosing technicians did not know the actual dosage or identity of any of the test groups. For the ptotic assays, the animals were randomized in respect to the six test groups of the 3 × 3 assay format, and also randomized between the two sexes, and between the two dosing technicians. The mean time off food prior to dosing for each group was statistically constant. Only one technician read the ptosis values for all animals at all indicated times, and this technician did not know the dosage level or identity of any of the test groups. Each mouse was permanently and individually identifiable throughout the test period due to spot applications of a 0.5% picric acid solution in 70% ethanol. Ptosis for each eye was scored following the scale adopted by Rubin, *et al.* (10): 4 = complete, 3 = 3/4, 2 = 1/2, 1 = 1/4 closure of the eyelids from the normal aperture. A nonptotic response was scored as 0. The individual metameter was the sum of the ptotic scores for both eyes, so that the graded test response varied from 0–8 per mouse. Prior to ptotic scoring, each mouse was manually aroused, then placed on a screen and held by the tail facing the scorer at the scorer's eye level. Eye scores of a 4 rating were checked to insure that the lid was not encrusted shut and that the ptosis was an active phenomenon.

Yohimbine hydrochloride and reserpine alkaloid were obtained from California Corporation for Biochemical Research. Reserpine was solubilized by glacial acetic acid and sufficient double-distilled water was added to make a 10 mg./ml. solution in 10% acetic acid. All dosages of reserpine and yohimbine are expressed here in terms of the alkaloid rather than the alkaloidal salt. Where

TABLE I.—MEAN BLEPHAROPTOTIC SCORES AFTER ORAL DOSAGE OF RESERPINE AND RESERPINE-YOHIMBINE MIXTURES TO MICE

Test Code S *vs.* U	Standard (S) Dosage, mg./Kg., Reserpine[a]	Standard (S) Hr. After Dosage 3	5	10	24	*vs.* Unknown (U) Dosage, mg./Kg.[a] Reserpine	Yohimbine	Unknown (U) Hr. After Dosage 3	5	10	24
R *vs.* R[1]:Y[0]	2	2.26[b]	3.61	3.92	1.18	2	..	2.07	3.41	3.61	1.33
	4	3.59	4.94	5.04	3.11	4	..	3.76	4.94	4.83	2.17
	8	4.65	6.52	7.09	6.41	8	..	5.31	6.74	7.35	6.81
R *vs.* R[1]:Y[1]	2	2.06[c]	3.25	3.44	1.78	2	2	3.14	3.86	3.28	1.36
	4	3.47	4.83	4.83	2.61	4	4	5.30	5.61	5.14	2.11
	8	5.50	6.89	7.67	6.72	8	8	6.64	7.25	7.44	5.17
R *vs.* R[1]:Y[2]	2	2.53[c]	3.78	4.06	0.78	1	2	2.83	3.67	3.11	0.94
	4	4.17	5.42	5.28	2.75	2	4	4.08	4.56	4.64	1.64
	8	4.92	6.67	7.25	6.78	4	8	5.08	5.92	5.72	3.03
R *vs.* R[1]:Y[4]	2	1.92[c]	3.50	3.80	1.22	1	4	2.75	3.50	2.72	0.86
	4	3.39	4.58	4.69	2.56	2	8	4.25	5.19	5.61	2.22
	8	4.53	6.33	6.75	6.33	4	16	5.44	6.06	5.75	3.80
R[F] *vs.* R[M]	2	1.87[d]	2.98	3.20	0.92	2	..	2.46	4.04	4.33	1.59
	4	3.57	4.68	4.62	2.50	4	..	3.78	5.20	5.24	2.78
	8	5.11	6.61	7.00	6.20	8	..	4.85	6.65	7.44	7.02

[a] The constant dosage volume for each test dose was 30 ml./Kg. [b] 54 mice per dosage group, equally divided between sexes. [c] 36 mice per dosage group, equally divided between sexes. [d] 54 mice per dosage group. The "standard" group is all female; the "unknown" group is all male.

yohimbine and reserpine were administered concurrently, they were given together in the same vehicle.

RESULTS AND DISCUSSION

Preliminary Evaluation of Yohimbine.—As calculated by the Bartlett modification of the Miller and Tainter log-probit method (13), the oral LD_{50} for yohimbine was determined to be 36.5 mg./Kg. with a standard error of ±3.02. The dosage increment used was 1.414×, and the number of animals (N') contributing to effects between probits 3.5 and 6.5 was 37. The estimated LD_5/LD_{95} values were 20.4/65.0 mg./Kg. Acute death from yohimbine was preceded by the following major symptoms listed in order of appearance: respiratory stimulation, respiratory depression, hyperkinetic phenomena (increased motor activity, fine body tremors, Straub tail erection), transient minimum ptosis (maximum rating of 1 per eye), clonic convulsions, cyanosis, and death by respiratory arrest. No evidence of priapism was seen in the males. The transient ptosis was seen only at dosage levels of 45.2 mg./Kg. or greater and was always seen prior to convulsions and death. Considering only quantal incidence of detectable ptosis, reserpine has a PtD_{50} (ptotic dose for 50% of a population) graphically estimated at 0.5 mg./Kg.; in this respect, reserpine is $\geq$128× the ptotic potency of yohimbine. Considered as a graded response, reserpine was graphically determined to be $\geq$64× the ptotic potency of yohimbine. Previous work in this laboratory has indicated that the acute oral LD_{50} of reserpine (acetate) was $\geq$150 mg./Kg. Therefore, the LD_{50}/PtD_{50} ratio for reserpine is $\geq$300; while for yohimbine, this ratio only equals 0.6. Yohimbine qualitatively displays little to no reserpine-like, sedative-ptotic activity.

Yohimbine Potentiation of Reserpine-Induced Palpebral Ptosis.—Four balanced log dose-response assays were conducted, assaying various reserpine-yohimbine mixtures against reserpine alone as a standard. The yohimbine content of the mixtures ranged from 0–4× the reserpine content. The maximum dose of yohimbine given with reserpine was 16 mg./Kg. Ptosis was rated for all test groups at 3, 5, 10, and 24 hours after dosing. Maximal mean ptotic effects after reserpine alone were seen 10 hours after dosing, in conflict with previous reports indicating that maximal effects were seen 5 to 7 hours after dosing (10). Mean results are tabulated in the first sections of Table I. Not reported in Table I are the mean ptotic scores of the 2 control groups receiving 8 and 16 mg./Kg. of yohimbine orally, respectively. Each group consisted of 18 mice, the number divided equally between the sexes, dosed and scored concurrently with the assay test groups. Mice receiving 8 mg./Kg. of yohimbine did not have detectable ptosis at any time, while the group receiving 16 mg./Kg. was observed to have a mean ptotic score of 0.18 and 0.12 at the 3- and 5-hour readings, respectively, with complete absence of ptosis at the 10- and 24-hour readings.

The statistical treatment for the 3 × 3 and 2 × 2 assays involved analysis of variance, factorial analysis, and calculation of potency and confidence limits as per the techniques outlined by Bliss (14). The results are tabulated in the first sections of Table II, with graphical interpretations illustrated in Figs. 1 and 2. An average λ value (s/b) of 0.29 was obtained with the 16 3 × 3 assays calculated; standard error was determined to be ±0.015. With this λ value, a population of 210 mice per assay would have been needed to insure a standard error for potency of ±10% (14). The number of mice used in this study was either 216 (36 mice/dose) or 324 (54 mice/dose) per each 3 × 3 assay.

Peak potentiation was noted at the third hour for 1:1 and 1:2 reserpine-yohimbine mixtures, while peak effects were reached with the tenth-hour readings with the 1:4 mixture (Fig. 1), indicating that at these concentrations and at these times, the presence of yohimbine in an unknown preparation will maximally distort any ptotic assay attempting to document only reserpine activity. The control

TABLE II.—DETERMINATION OF APPARENT POTENCIES OF TEST UNKNOWNS IN RELATION TO RESERPINE ALONE OR TO RESERPINE IN FEMALE MICE

S *vs.* U Test Code from Table I	Hr. After Oral Dosage 3	5	10	24
R *vs.* R[1]:Y[0]	1.112[a] (0.933–1.326) 0.25–0.20[b]	1.003 (0.869–1.157) >0.50[b]	0.966[c] (0.847–1.102) >0.50[b]	0.967[cd] (0.865–1.081) >0.50[b]
R *vs.* R[1]:Y[1]	1.715 (1.418–2.075) <0.001[b]	1.259 (1.088–1.456) 0.005–0.001[b]	0.991 (0.874–1.124) >0.50[b]	0.820[e] (0.716–0.939) 0.005–0.001[b]
R *vs.* R[1]:Y[2]	2.161 (1.737–2.689)	1.467 (1.203–1.790)	1.219 (1.006–1.477)	1.265[e] (0.929–1.722)
R *vs.* R[1]:Y[4]	1.576 (1.245–1.994)	2.118 (1.716–2.613)	2.586[e] (2.161–3.095)	1.707[e] (1.129–2.580)
R(F) *vs.* R(M)	1.092 (0.917–1.301) 0.50–0.25[b]	1.269[f] (1.098–1.467) <0.001[b] 1.462[e] (1.188–1.800) <0.001[b]	1.340[c] (1.170–1.534) <0.001[b] 1.174[e] (1.036–1.330) 0.01–0.005[b]	1.164[c] (1.040–1.303) 0.01–0.005[b] 1.100[e] (0.991–1.221) 0.10–0.05[b]

[a] Figures in parentheses indicate the 95% confidence limits for the potency. [b] Observed *P* of test of S *vs.* U. [c] Significant curvature in 3 × 3 assay, *P* 0.05. [d] Significant opposite curvature in 3 × 3 assay, *P* 0.05. [e] Calculated as a 2 × 2 assay. [f] Significant departure from parallelism in 3 × 3 assay, *P* 0.05.

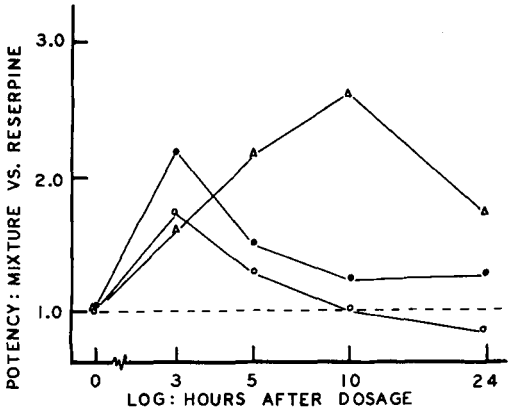


Fig. 1.—Variation with time of ptotic potency of various reserpine-yohimbine mixtures: O, 1:1 reserpine-yohimbine; ●, 1:2 reserpine-yohimbine; △, 1:4 reserpine-yohimbine.

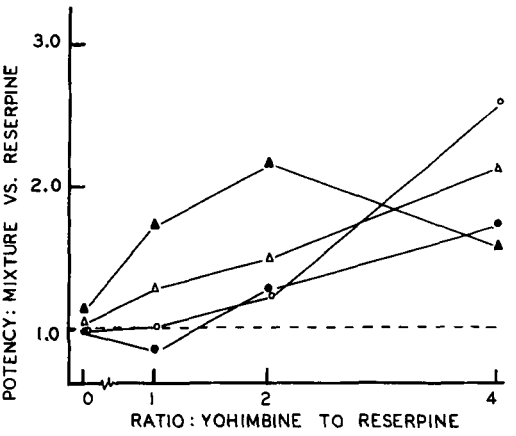


Fig. 2.—Variation of ptotic potency with ratio of yohimbine to reserpine in reserpine-yohimbine mixtures: ▲, 3-hour ptosis response; △, 5-hour; O, 10-hour; ●, 24-hour.

assay with no yohimbine in the unknown showed no potentiation at any of the time intervals.

Yohimbine in a 1:1 ratio in a reserpine-yohimbine mixture potentiated the 3- and 5-hour ptosis readings, had no effect on the 10th-hour ptosis, and may have been actually instrumental in reversing the residual 24-hour reserpine ptosis (Fig. 2). Yohimbine, in a ratio of 1:2 in reserpine-yohimbine mixtures, potentiated reserpine on the 3-, 5-, and 10-hour ptotic responses, but the effect on the 24-hour ptosis was not statistically significant. Yohimbine potentiated at all observed time intervals when in a 1:4 reserpine-yohimbine mixture indicating that in high levels, yohimbine is capable of exerting considerable sustained effects.

Sex Variation in Response to Reserpine.—In the preceding assays a consistent sex variation to the reserpine-induced blepharoptotic response was detected, so a factorially designed assay was performed to document this observation. The results are tabulated in the last part of Table I, and the statistical evaluation noted in the last lines of Table II. At 5, 10, and 24 hours after dosing with reserpine alone, the males were significantly more susceptible than the females, with the greatest sensitivity being apparent at the 5- and 10-hour ptotic readings.

CONCLUSIONS

Yohimbine, although lacking true ptotic activity, potentiated reserpine-induced blepharoptosis without altering slope, dose-response relationships of this phenomenon, thus indicating that the mechanism of action of reserpine may involve depletion of norepinephrine more than the release of serotonin as a neurohormone. In mice, the males are more susceptible to oral

reserpine than the females. The presence of yohimbine in crude plant material may distort results obtained by mouse blepharoptotic assays where the goal of the assay is the quantitation or the detection of only reserpine-like alkaloids.

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Synthesis and Pharmacological Effects of Bis-trialkylammonium Alkanol Carbonates

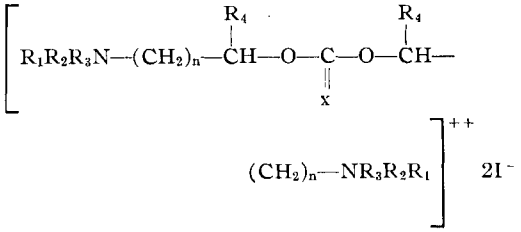
By LEO A. POHORYLES, L. WISLICKI, and SHALOM SAREL

The synthesis and pharmacological effects of a series of bis-trialkylammonium alkanol carbonates of the type I, are described. The compounds were tested in the cat for their effects on electrical excitability of striated muscle, respiration, and blood pressure, and in the frog rectus abdominis. While the ethyl derivative, (I-1), lowers blood pressure without affecting the muscle twitch, the propyl-(I-3) and, to a much higher extent, the butyl derivative, (I-6), depress neuromuscular transmission and also direct muscle excitability. Branching of the alkanol moiety (I-2), or replacement of the carbonate oxygen by sulfur (I-7), or insertion of ethyl for the methyl groups on the quaternary nitrogen (I-4 and I-5), weaken the effect. The block is similar to that caused by depolarizing agents.

IT IS WELL known that the curarizing activity of bis-quaternary ammonium compounds of the structure $R_3\overset{+}{N}—(CH_2)_x—\overset{+}{N}R_3$ is not substantially weakened by inserting in the linear polymethylene chain bivalent radicals like carbonyl and/or oxygen for the methylene groups. Introduction of one or more ester groupings into the chain linking the two ammonium groups provides powerful curare-like neuromuscular blocking agents; thus, choline esters of dicarboxylic acids, particularly that of succinic acid, proved to be valuable clinically. However, these substances were found to be not without drawbacks. These include, for the nondepolarizing drugs, their long action and side effects on heart rate or blood pressure. With suxamethonium, which *in vivo* is usually quickly decomposed by enzymatic hydrolysis, a high incidence of muscle pain (1, 2), the absence of an effective antagonist, and instability within a range of pH's (3) have been troublesome.

Received June 21, 1961, from the Hebrew University-Hadassah Medical School, Jerusalem, Israel.
Accepted for publication August 4, 1961.

In view of the great stability shown by open chain carbonates toward hydrolysis both in acid and alkaline solutions (4), the synthesis of a series of bis-trialkylammonium alkanol carbonates of the structure I was undertaken.



I

I-1 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 1$; $x = O$.
I-2 $R_1 = R_2 = R_3 = R_4 = CH_3$; $n = 1$; $x = O$.
I-3 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 2$; $x = O$.
I-4 $R_1 = R_2 = CH_3$, $R_3 = C_2H_5$, $R_4 = H$, $n = 2$, $x = O$.
I-5 $R_1 = R_2 = C_2H_5$; $R_3 = CH_3$; $R_4 = H$; $n = 2$; $x = O$.
I-6 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 3$; $x = O$.
I-7 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 3$; $x = S$.

Differences in structure were designed with a

reserpine than the females. The presence of yohimbine in crude plant material may distort results obtained by mouse blepharoptotic assays where the goal of the assay is the quantitation or the detection of only reserpine-like alkaloids.

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Synthesis and Pharmacological Effects of Bis-trialkylammonium Alkanol Carbonates

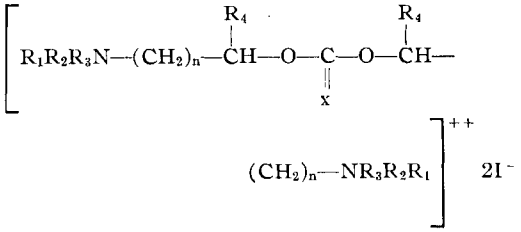
By LEO A. POHORYLES, L. WISLICKI, and SHALOM SAREL

The synthesis and pharmacological effects of a series of bis-trialkylammonium alkanol carbonates of the type I, are described. The compounds were tested in the cat for their effects on electrical excitability of striated muscle, respiration, and blood pressure, and in the frog rectus abdominis. While the ethyl derivative, (I-1), lowers blood pressure without affecting the muscle twitch, the propyl-(I-3) and, to a much higher extent, the butyl derivative, (I-6), depress neuromuscular transmission and also direct muscle excitability. Branching of the alkanol moiety (I-2), or replacement of the carbonate oxygen by sulfur (I-7), or insertion of ethyl for the methyl groups on the quaternary nitrogen (I-4 and I-5), weaken the effect. The block is similar to that caused by depolarizing agents.

IT IS WELL known that the curarizing activity of bis-quaternary ammonium compounds of the structure $R_3\overset{+}{N}—(CH_2)_x—\overset{+}{N}R_3$ is not substantially weakened by inserting in the linear polymethylene chain bivalent radicals like carbonyl and/or oxygen for the methylene groups. Introduction of one or more ester groupings into the chain linking the two ammonium groups provides powerful curare-like neuromuscular blocking agents; thus, choline esters of dicarboxylic acids, particularly that of succinic acid, proved to be valuable clinically. However, these substances were found to be not without drawbacks. These include, for the nondepolarizing drugs, their long action and side effects on heart rate or blood pressure. With suxamethonium, which *in vivo* is usually quickly decomposed by enzymatic hydrolysis, a high incidence of muscle pain (1, 2), the absence of an effective antagonist, and instability within a range of pH's (3) have been troublesome.

Received June 21, 1961, from the Hebrew University-Hadassah Medical School, Jerusalem, Israel.
Accepted for publication August 4, 1961.

In view of the great stability shown by open chain carbonates toward hydrolysis both in acid and alkaline solutions (4), the synthesis of a series of bis-trialkylammonium alkanol carbonates of the structure I was undertaken.



I

I-1 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 1$; $x = O$.
I-2 $R_1 = R_2 = R_3 = R_4 = CH_3$; $n = 1$; $x = O$.
I-3 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 2$; $x = O$.
I-4 $R_1 = R_2 = CH_3$, $R_3 = C_2H_5$, $R_4 = H$, $n = 2$, $x = O$.
I-5 $R_1 = R_2 = C_2H_5$; $R_3 = CH_3$; $R_4 = H$; $n = 2$; $x = O$.
I-6 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 3$; $x = O$.
I-7 $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 3$; $x = S$.

Differences in structure were designed with a

view toward studying their effect on the pharmacological pattern. As expected, the new quaternary ammonium iodides reported here exhibited remarkable stability in water solutions for a long period of time and could even be sterilized at 80° for 2 hours without chemical decomposition or changes in their pharmacodynamic effect.

EXPERIMENTAL PROCEDURE[1]

Materials.—The sources and purification procedures for the dialkylaminoalkanols used were as follows: commercial dimethylaminoethanol was fractionated and the cut b_{690} 130–133°, n_D^{22} 1.4280 was used. The 3-diethylamino-1-propanol (Eastman Chemical Co.) was fractionally distilled and the cut b_{23} 91–93° was collected. The 3-dimethylamino-1-propanol (Light's Organic Chemicals) was fractionated and the fraction b_{690} 185–190° was used. Thiophosgene (Matheson Coleman and Bell) was used without purification.

1-Dimethylamino-2-propanol was prepared in 30% yield by reacting dimethylamine with propylene oxide following Goldfarb's procedure,[5] b_{690} 116–119° (reported (5) b_{758} 124.5–126°), n_D^{17} 1.4115.

4-Chloro-1-butanol, b_4 62–66°, n_D^{20} 1.4490, was prepared in 50% yield following the method of Starr and Hixon (6).

4-Dimethylamino-1-butanol was prepared by reacting 4-chloro-1-butanol with aqueous dimethylamine at elevated temperature in a sealed system, as described below: a mixture of 4-chloro-1-butanol (109 Gm., 1 mole) and 30% aqueous dimethylamine (90 Gm., 2 moles) was placed in an autoclave and then gradually heated to 150°, causing the pressure to rise to 20 Atm. After 6 hours, heating was stopped and the reaction mixture was continuously extracted with ether during 4 days. The desired dimethylamino-butanol was isolated and purified by its fractionation through an efficient column, giving 56 Gm. (50% yield), of a colorless oil b_{20} 90–93°, n_D^{20} 1.4405 (Avison (7) reported b_{12} 78°, and Breckpot (8) reported n_D^{20} 1.4408).

The synthetic method used in this study involved: (*a*) the phosgenation of the appropriate ω-dialkylaminoalkanol (the resulting bis-dialkylaminoalkyl carbonate being not always isolated), followed by (*b*) quaternization of the corresponding base by means of methyl iodide.

Preparation of Bis-trialkylammonium-alkyl Carbonate Halogenides.—In a typical experiment, a cooled solution of 27 Gm. (0.27 mole) of phosgene in 135 ml. dry toluene was added with stirring to a solution of 4-dimethylamino-1-butanol (58.4 Gm., 0.4 mole) in dry toluene (100 ml.) at 0°, at such a rate that the temperature of the reaction mixture was maintained at 14 to 20°. After the addition was completed, the reaction mixture was stirred for an additional 2 hours, giving a heavy hygroscopic white precipitate (72 Gm.) of the required ester dihydrochloride. It was collected by quick filtration at the water pump, washed with dry ether, and then dissolved in dry ethanol. The free bis-dimethylaminobutyl carbonate was obtained by saturating the above ethanolic solution with dry gaseous ammonia at 0°. The free base, which is very soluble in ethanol, remained in the solvent; whereas the insoluble ammonium chloride separated, giving a white crystalline precipitate. Excess of ammonia was first removed by passing through the reaction mixture a stream of dry nitrogen and then the precipitate of ammonium chloride was removed by filtration. The free base was used in the subsequent step without being isolated.

Bis-trimethylammonium-butyl Carbonate Iodide.—To the resulting solution of bis-dimethylaminobutyl carbonate in ethanol (500 ml.) described above, there was added dropwise, with shaking, methyl iodide (80 Gm.) at such a rate that the temperature of the reacting system was maintained at 25°. After addition was completed, the reaction mixture was heated at 40° for 2 hours and then it was concentrated to half its volume by evaporating the solvent at reduced pressure. On cooling, the required bis-trimethylammonium-butyl carbonate iodide separated as a semisolid product. The purification of the bis-quaternary ammonium salt was effected by the dropwise addition of either *n*-butanol or dry ether to its stirred methanolic solution, providing 25 Gm. of the pure compound as a white powder.

Bis-trimethylammonium-butyl Thiocarbonate Iodide.—A solution of 4-dimethylaminobutanol-1 (20 Gm., 0.17 mole) in 100 ml. dry toluene was reacted with thiophosgene (10 Gm., 0.087 mole) dissolved in 50 ml. toluene in the fashion described above. After removing half of the amount of solvent by distillation at reduced pressure, the thiocarbonate derivative thus formed was not isolated but reacted with an excess of methyl iodide. The desired quaternary ammonium iodide could not be induced to recrystallize from ordinary solvents; it was purified by its repeated dissolution in methanol and precipitation by adding ether.

The melting points and yields of the compounds prepared are given in Table I.

PHARMACOLOGY

The experiments were performed on cats under pentobarbital anesthesia following induction with ether; a total of 34 animals was used. An external jugular vein was cannulated for injections, and a femoral or carotid artery for measurement of blood pressure. Respiration was recorded by means of a tambour connected to a tracheal cannula which was fitted with an adjustable air leak.

To test muscle excitability, an Achilles tendon was separated from the bone and connected to an isometric lever. The ipsilateral sciatic nerve was divided high in the thigh and its distal portion mounted on a pair of shielded silver electrodes, the distal electrode serving as cathode. The anode of a second pair of electrodes was attached to the gastrocnemius near its origin and the cathode placed on its tendon. Supramaximal square wave stimuli were administered with a Fleming stimulator; pulse width of indirect stimuli was 0.15 msec. and that of direct stimuli 1.2 msec. By means of relays, indirect and direct stimuli could be delivered alternately; they were applied either at a rate of 15/min. or in pairs, an indirect stimulus preceding a direct

[1] All m.p.'s were taken on Dr. Tottoli melting point apparatus and are uncorrected.

TABLE I.—PROPERTIES OF SOME BIS-TRIALKYLAMMONIUM-ALKYL CARBONATE DI-IODIDES

No.		M.p., °C.	Yield, %		Calculated C	Calculated H	Calculated I	Calculated N	Found C	Found H	Found I	Found N
1	Bis-trimethylammonium-ethyl carbonate	203–205	50–60	$C_{11}H_{26}I_2N_2O_3$	27.0	5.33	52.0	5.74	27.32	5.49	51.2	6.1
2	Bis-1-trimethylammonium-2-propyl carbonate	242	18	$C_{13}H_{30}I_2N_2O_3$	30.2	5.81	49.22	5.42	30.3	5.88	50.37	5.15
3	Bis-1-trimethylammonium-3-propyl carbonate	166–167	65	$C_{13}H_{30}I_2N_2O_3$	30.2	5.81	49.22	5.42	29.6	5.77	48.9	5.68
4	Bis-1-dimethylethylammonium-3-propyl carbonate	189	40	$C_{15}H_{34}I_2N_2O_3$	33.1	6.25	46.7	5.15	32.0	6.66	45.76	5.07
5	Bis-1-diethylmethylammonium-3-propyl carbonate	197–199	60	$C_{17}H_{38}I_2N_2O_3$	35.66	6.64	44.4	4.49	35.82	7.08	43.33	4.61
6	Bis-1-trimethylammonium-4-butyl carbonate	186	57	$C_{15}H_{34}I_2N_2O_3$	33.1	6.25	46.7	5.15	33.35	6.15	46.0	5.18
7	Bis-1-trimethylammonium-4-butyl thiocarbonate	280 (decompn.)	..	$C_{15}H_{34}I_2N_2O_2S$	32.0	6.10	45.3	5.0	31.80	6.28	46.0	5.3

one by 2 seconds and each pair being separated from the next by 6 seconds.

For preparations of the rectus abdominis muscle, *Rana ridibunda* was used.

RESULTS

The lower homolog in the series, compound I-1, in which the ammonium groups are separated by a linear chain of seven atoms, did not affect muscle excitability, but 0.2–0.5 mg./Kg., given intravenously, reduced blood pressure by 40–55 mg. Hg for a few minutes. Intramuscular injection of 16 mg./Kg. was ineffective (3 observations).

With the next higher homolog in this series, compound I-3, in which the ammonium groups are separated by a linear carbonate chain of nine atoms, inhibition of muscle excitability appeared while the hypotensive action receded (2 observations). As Fig. 1 shows, 0.3 mg./Kg. i.v. depressed neuromuscular transmission and, to a lesser extent, the response to direct stimulation and respiratory movements; 1 mg./Kg. abolished indirect excitability for 75 minutes, halved the response to direct stimulation for 50 minutes, and caused apnea for one-half hour.

The blocking effect on muscle was strongly enhanced in I-6 (16 observations). Sensitivity of the animals to this substance varied greatly: usually

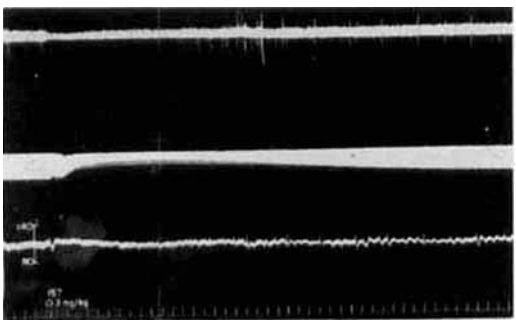


Fig. 1.—Cat, ether-pentobarbital anesthesia. Alternating direct and indirect supramaximal stimulation of gastrocnemius muscle. From top down: respiration, gastrocnemius twitch, blood pressure, time, in minutes, and signal. Effect of 0.3 mg./Kg. I-3 i.v.

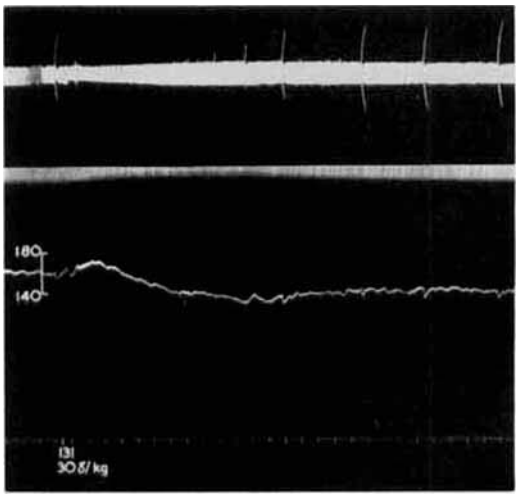


Fig. 2.—Arrangement as in Fig. 1. Effect of 30 mcg./Kg I-6 i.v.

15–30 mcg./Kg. produced only a partial block in the gastrocnemius and very slight depression of respiration (Fig. 2); 40 mcg./Kg. (Fig. 3) caused, after fast injection, potentiation of the twitch response and general fasciculations which lasted for a few minutes; neuromuscular transmission and spontaneous respiration were abolished and direct muscle excitability depressed for longer periods. Recovery of respiration was much quicker than that of the leg muscle.

Neostigmine did not restore neuromuscular transmission though two intravenous injections of 30 mcg./Kg. each, administered within 1 minute, stimulated respiration and speeded up return of excitability slightly.

The onset of the block after substance I-6 was similar to that after depolarizing agents, e.g., suxamethonium, in that it was characterized by augmentation of twitch height and by fasciculations; it also caused in the frog rectus abdominis a contraction whose speed rose with the dose administered without, however, approaching that observed after similar doses of acetylcholine (Fig. 4); tachyphylaxis did not appear with the amounts used.

The thio-derivative of the carbonic acid, I-7, proved to be about 100 times less effective as a

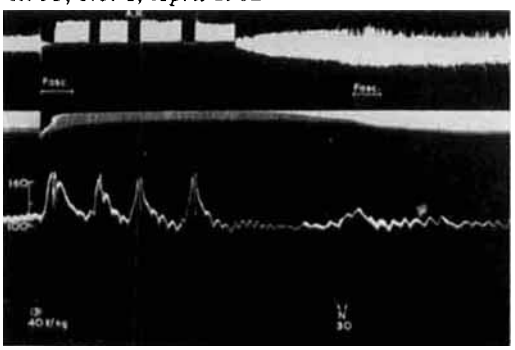


Fig. 3.—Arrangement as in Fig. 1. A.R., artificial respiration; Fasc., general fasciculations; N, 30 mcg./Kg. neostigmine i.v.; effect of 40 mcg./Kg. I-6 i.v.

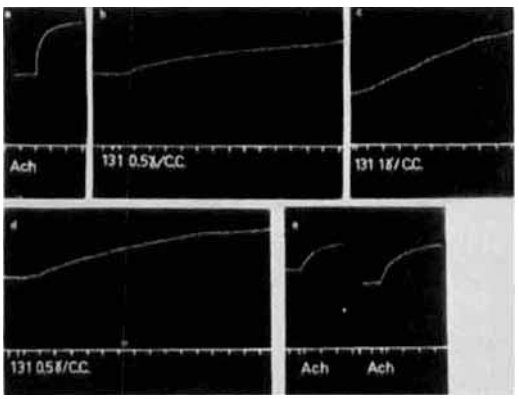


Fig. 4.—Effects of 0.5–1 mcg./ml. I-6 on frog rectus abdominis muscle compared to that of 0.7 mcg./ml. acetylcholine (Ach). Intervals between curves, 5 minutes when the muscle was washed three times. Time in min.

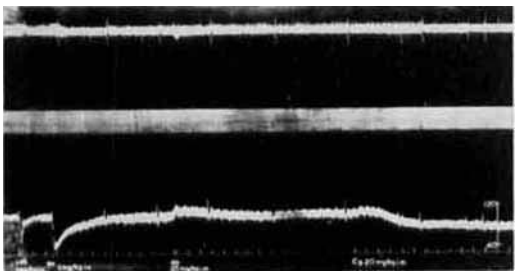


Fig. 5.—Arrangement as in Fig. 1. Effect of 1 and 5 mg./Kg. I-2 i.v. and of 25 mg./Kg. I-2 i.m. compared to that of 20 mg./Kg. hexamethonium i.m.

blocking agent than the parent compound (3 observations). About 3 mg./Kg. were required to produce effects on muscle excitability, respiration, and blood pressure similar to those observed after 15–40 mcg./Kg. of I-6.

Unlike lengthening of the chain in the linear homologs of the series, branching at the carbons linked to the oxygens resulted in compounds with diminished potency. Thus, 1–5 mg./Kg. of I-2, given i.v., produced a brief hypotension, but 25 mg./Kg., injected i.m., did not lower blood pressure (4 observations). In the experiment shown in Fig. 5 these effects are compared with that of hexamethonium. Substitution in the quaternary ammonium group of methyls by ethyls also weakened the biological actions. Thus I-4, which has one ethyl group linked to each ammonium group, had a shorter paralyzing action than the trimethyl analog, and the di-ethyl methyl analog (I-5) was even ten times less effective (3 observations).

DISCUSSION

The weak hypotensive action of I-1 and the absence of this effect in I-3 is in keeping with the observations made by Paton and Zaimis (9) on the cat's superior cervical ganglion.

As far as neuromuscular blocking potency was concerned, Paton and Zaimis' curve shows highest activity for decamethonium (C_{10}) and a steep fall for longer chain substances; for the series reported in this paper, the distance of 11 atoms resulted in a compound which was 10–20 times more powerful in depressing muscle excitability than the one whose chain was shorter by two carbons.

The decreasing tendency in curarizing action resulting either from substituting methyls for ethyls on the quaternary nitrogen or from branching of the polymethylene chain corresponds to that reported by Bovet and co-workers (10).

Augmentation of the twitch and general fasciculations which frequently appear immediately after injection of I-6 indicate that the block caused by this substance is of the depolarizing type. A comparable action is the failure of neostigmine to antagonize the block, which conforms to that described for decamethonium, and the stimulating action on the frog rectus abdominis, which corresponds to that of suxamethonium and other depolarizing agents (9, 11).

Unlike suxamethonium which undergoes partial hydrolysis upon keeping its aqueous solutions at room temperature (12), I-6 was found to be stable for at least 1 year under comparable conditions.

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Preliminary Phytochemical Study of *Lychnis alba* Saponin

By KARLIS K. KAZEROVSKIS

Distribution of *Lychnis alba* saponin in the plant is determined. A method for the isolation of the saponin by column chromatography and the detection of the saponin on paper chromatograms with a gelatin blood suspension gel is reported.

SAPONIN containing plants have been used in therapeutics and in the household for centuries. In recent years, these plants have become a focal point in pharmaceutical research. Discovery of the adrenal cortical steroids resulted in a large scale and systematic investigation of plants containing sterol saponins in the United States as possible sources of the precursors essential to the synthesis of cortisone and its derivatives. U. S. Department of Agriculture research groups (1) have tested about 5,000 samples representing a large number of the genera and plant families of the United States and abroad for their saponin, alkaloid, flavanoid, and sterol content. In an earlier phytochemical study (2), 21 wild growing plants in Latvia were tested for the presence of saponins.

In the phytochemical studies of wild growing plants in Michigan, *Lychnis alba* Mill., a member of the family *Caryophyllaceae*, seemed to deserve a closer investigation due to its hemolytic properties, abundance, and relationship to *Saponaria officinalis* L., a well-known source of a saponin. *Radix saponariae* is used in Germany as an expectorant (3).

EXPERIMENTAL

Plant.—*Lychnis alba* Mill. (Fig. 1) known also as *Melandrium album* Rydb. was originally described by Miller. This plant is also known as white cockle, campion, and evening lychnis (4).

The morphological description of the plants collected for the investigation were checked (4, 5) and identification of the plants was confirmed by Drs. Virginia and Harold E. Bailey of Wayne State University, Detroit, Mich.

Lychnis alba Mill. is a naturalized plant from Eurasia growing on the waste places, along the roadsides and borders of the fields, and on the recently idled farm lands. The Northern boundary of its distribution extends from Quebec to British Columbia, and to the South as far as North Carolina, Ohio, Illinois, Colorado, and California.

It is a dioecious annual or short-lived perennial plant, 2 to 3 feet tall, with a stout root system. Inflorescence is usually branched. Flowers are white, fragrant, and opening in the evening. Fruit is an ovoid capsule.



Fig. 1.—*Lychnis alba* Mill. staminate and pistillate plants.

Material and Method.—The plant material was collected in Mecosta County, Mich., in June and October 1959, dried in a drying oven, and powdered to pass through a No. 40 mesh.

Distribution of the saponin in the plant was determined by Kobert's hemolytic index (6) see Table I. Citrated rat blood was used. Plant extracts were prepared in a normal saline solution on a steam bath.

In this study 2 Kg. of the dried roots were used. Fatty and waxy substances were removed with petroleum ether (b.p. 30–60°) and ether. Hemolysis was used as a test to follow the course of the isolation of the saponin.

Solubility and heat stability tests of *L. alba* saponin indicated that the saponin could be ex-

Received May 24, 1961, from the Division of Pharmacy, Ferris Institute, Big Rapids, Mich.
Accepted for publication June 23, 1961.
This study was supported by an Upjohn Research Grant and technically assisted by Malcolm MacKenzie and Carlton K. Erickson, pharmacy seniors in the Division of Pharmacy.
Presented to the annual meeting of the American Society of Pharmacognosy, Boulder, Colorado, June 1960.

TABLE I.—HEMOLYTIC INDEXES OF PARTS OF *L. alba*

Part Used	Concn. of Saline Extract Prepared	Hemolytic Index
Root	1:250	1250
Seeds	1: 50	166
Leaves	1: 50	166
Flowering tops	1: 50	did not hemolyze

tracted by hot water or hot alcohol. From the hot 95% alcoholic extract, a part of the saponin precipitates as a white amorphous substance. Preliminary adsorption tests on alumina and Florisil columns revealed that *L. alba* saponin is adsorbed on the alumina as well as on the Florisil when water or 95% alcohol is used as a solvent. From developed chromatograms the saponin could be eluted with a 25% alcohol.

Three fractions of *Lychnis alba* saponin (fractions A, B, and C) were prepared in the following manner:

Fraction A.—A 1-Kg. portion of the dried powdered root was extracted with water on a steam bath four times for 4 hours each time. Saponin fraction was adsorbed on a Florisil column and eluted with 25% alcohol. After removal of the solvent in a flash evaporator, an amorphous, slightly yellowish substance was obtained, fraction A. Hemolytic index of this fraction was 13,300.

Fraction B.—Dried powdered root, 1 Kg., was extracted with 95% alcohol on a steam bath six times for 4 hours each time. From the hot alcoholic extract, a part of the saponin precipitated out, fraction B. Hemolytic index of this fraction was 6,600.

Fraction C.—That saponin fraction remaining in the solution of 95% alcohol was again adsorbed on a Florisil column and eluted with 25% alcohol. After removal of the solvent, an amorphous yellowish substance was obtained, fraction C. Hemolytic index of this fraction was 6,600.

All efforts to crystallize the saponin fractions failed. The same was true in using cholesterol for purification.

Paper Chromatography.—In available literature glycosides as well as saponins have been detected on the paper chromatograms by using reagents forming colored spots with the glycone of the glycoside, or specific reagents are used to detect the aglycone.

Bliss and Ramstad (7) used paper chromatography for the glycoside separation and located the glycosides on the chromatograms after hydrolysis with carbohydrases and spotting the sugars with an aniline hydrogen phthalate reagent. Dutta (8) has used a sodium *meta*-periodate in alkaline potassium permanganate solution to identify the saponin spots on paper chromatograms.

Gelatin blood suspension in an isotonic solution has been used for a long time for the microchemical detection of the saponins in the plant materials as indicated by Fischer (9).

In this study a gelatin blood suspension gel was used to reveal the saponin spots on the paper chromatograms.

Strips of Whatman filter paper No. 1 were spotted with a 2% solution (5 λ) of saponin fractions A, B, and C, then dried and placed in a chromatographic chamber. When the equilibrium in the chamber was reached, chromatograms were developed with several developers. Gelatin blood suspension (5 Gm. gelatin, bacteriology grade, was dissolved in an isotonic solution containing 0.7% sodium chloride and 0.6% secondary sodium phosphate; to 100 ml. of such a solution, 5 ml. of citrated rat blood was added) was poured through muslin, to retain the air bubbles, on a leveled glass plate on which the desired area was enclosed with melted paraffin.

In order to provide a closed chamber around the blood gelatin suspension, another glass plate was placed on rubber tubing surrounding the designated area. When gel was formed, paper chromatograms were placed on the gel. After 2 to 4 hours, red blood cells hemolyzed under the saponin spots on paper chromatograms and the filter paper in that area stained reddish-brown. See Table II and Fig. 2.

TABLE II.—R_f VALUES OF THE SEPARATE FRACTIONS OF *L. alba* SAPONIN USING ASCENDING CHROMATOGRAPHY

Developer	Fraction A	Fraction B	Fraction C
No. 1 *n*-Butanol, 60 ml. Ethanol, 13 ml. 1 *N* Ammonia, 27 ml.	0.33	0.33	0.33
No. 2 *n*-Butanol saturated with 1 *N* HCl	0.14	0.14	0.14
No. 3 *n*-Butanol, 4 ml. Acetic acid, 1 ml. Distilled water, 5 ml.	0.15	0.16	0.16
No. 4 *n*-Butanol saturated with water	0.09	0.11	0.10

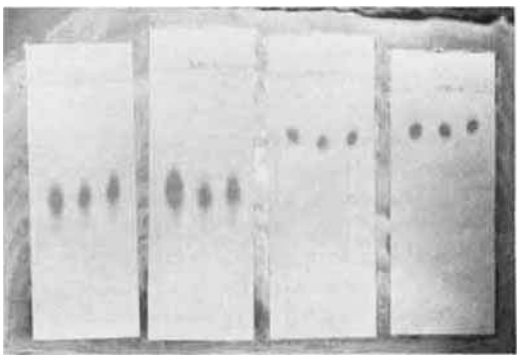


Fig. 2.—Saponin spots on paper chromatograms after hemolysis. The first two chromatograms are digitonin, the second two chromatograms are *Lychnis alba* saponin.

As long as developer No. 1 provided the highest R_f value for *L. Alba* saponin, several experiments were performed by spotting wide paper strips with all three saponin fractions and developing by developer No. 1. According to the results obtained, each of the three fractions contains one saponin and it is likely the saponin in each fraction is the same compound.

SUMMARY

Lychnis alba Mill. is a plant which contains a saponin. Most of the saponin is in the root.

Saponin can be extracted with hot water or hot alcohol, concentrated and purified on a Florisil column, and eluted with 25% alcohol.

Three fractions of *L. alba* saponin have been prepared and it is very likely that they contain just one saponin.

Gelatin blood suspension gel is useful in the detection of saponin on paper chromatograms.

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(1) Wall, M. E., *et al.*, THIS JOURNAL, **48**, 695(1959).
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Kinetics of Thiamine Hydrolysis

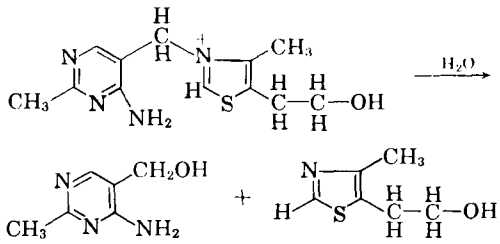
By JOHN J. WINDHEUSER and TAKERU HIGUCHI

The pH-rate profile of the hydrolytic cleavage of thiamine determined from kinetics measurements has been found to be unusually complex. Under essentially buffer-free conditions, the overall behavior suggests existence of at least four separate reactions: (*a*) acid catalyzed mechanism leading to the formation of oxythiamine at pH below 1; (*b*) water cleavage of protonated thiamine yielding a pyrimidine and a thiazole fraction between pH 1–6; (*c*) at pH 2–6.5, hydroxyl ion catalysis of protonated thiamine producing the same products found in reaction (*b*); (*d*) above pH 6.5, water cleavage of thiol thiamine to yield a pyrimidine diamine and other undetermined products. The ionic strength and temperature dependency of the reaction has also been determined. Contrary to earlier works, the hydrolytic reaction appears not to be subject to major negative or positive catalysis by amino acid species. The rate, however, is pronouncedly influenced by what appears to be general base catalysis by several buffer species.

ALTHOUGH hydrolytic cleavage of thiamine, an important pharmaceutical product, represents the major mode of anaerobic degradation of the vitamin, the kinetic dependency of this reaction on pH has not previously been reported in terms of the reactive species present. The present contribution is concerned with the results of an investigation designed to establish the pH profile of this important reaction, to reconfirm the degradation products under the conditions of the experiments and to correlate them with the kinetic observations, to determine whether agents other than hydroxyl and hydrogen ions can accelerate or inhibit the cleavage, and to establish its dependency on other kinetic variables.

Some doubt has been expressed previously on the question of whether the cleavage followed a strictly first-order kinetic expression with respect to the vitamin (1–3), although it appears generally agreed that the rate of hydrolysis was enhanced by increasing pH and the temperature (4–8). Others (9, 10) have also noted that buffers increased the rate of the reaction, although no attempts were made to evaluate the specific effects. McIntire and Frost (2), studying the effects of various compounds on the hydrolytic rate, claimed to have found inhibitory and catalytic effects but did not establish mechanisms for the observed results.

Several cleavage products have been recovered and identified. Watanabe (11), working with the reaction products resulting from the hydrolysis of a 0.5% thiamine solution heated at 140° for 12 hours at approximately pH 3.5, the pH resulting from a solution of the salt itself, was able to isolate and identify the degradation products and proposed the following reaction



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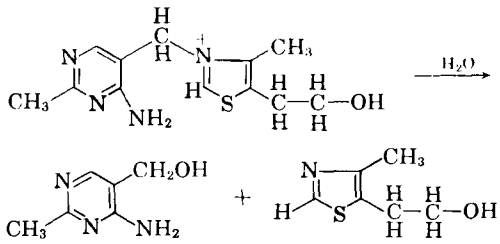
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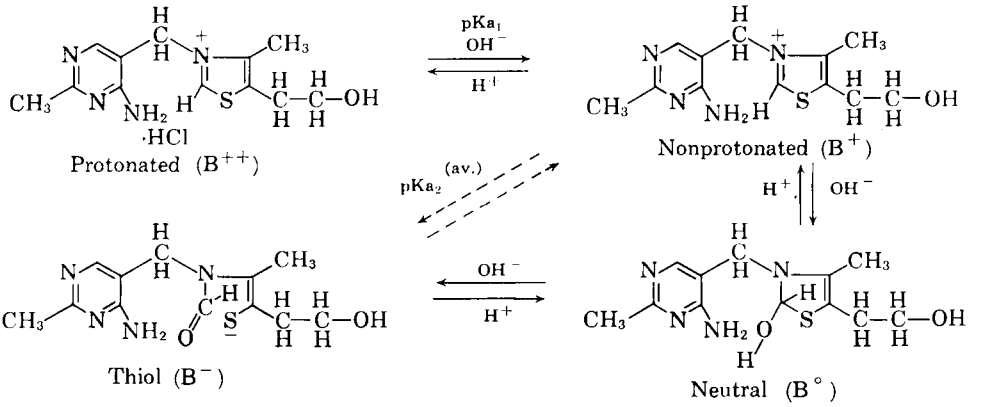
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In a recent publication, Watanabe (12), reviewing the physical chemical properties of thiamine, indicated that in alkaline solution the products of the degradation became more complex and differed from those found in the acidic region. Under strongly acidic conditions the products of the degradation again change. Rydon (13) was able to prepare and isolate oxythiamine by refluxing thiamine in 6 *N* hydrochloric acid for 6 hours. This compound appeared to be formed by the hydrolysis of the 4-amino group on the pyrimidine ring.

Any detailed kinetic analysis of the hydrolytic rate must take into account the existence of different thiamine species in solution. The vitamin undergoes several transformations under conditions of increasing pH. Williams (14) found, when titrating thiamine chloride hydrochloride with base, that after one mole equivalent of base, the pH did not continue to rise rapidly on further addition. The addition of two more mole equivalents of base were required for complete neutralization. He further observed that the last two moles of base reacted at a finite rate, indicating an internal rearrangement, which he postulated as follows



Back titration with acid showed the reaction to be reversible. As it was not possible to determine the concentration of the neutral intermediate, it was necessary to calculate an average equilibrium constant for the conversion of the nonprotonated thiamine to the thiol form, rather than the constants for the individual steps. This constant was designated as the average second dissociation constant. From his data, Williams calculated pKa_1 to be 4.8 and pKa_2 to be 9.0 at 25°. These values are in good agreement with those found by later workers (15, 16).

RESULTS AND DISCUSSION

Reaction Order with Respect to Thiamine.—The results of the present investigation clearly indicate that the hydrolytic reaction proceeds at rates directly proportional to the thiamine concentration in accord with the recent suggestion of Watanabe (12) and in contrast to his earlier work (1) and that of Farrer (3) and McIntire and Frost (2). A plot of the logarithm of the residual thiamine concentration *vs.* reaction time yielded a straight line in each case. The calculated first-order rate constants were found to be identical within experimental limitations. The observed half-lives of the reaction at varying thiamine concentrations are shown in Table I. For our studies, solutions were prepared at constant ionic strength, constant buffer concentration, constant pH, but with varying amounts of thiamine.

TABLE I.—EFFECT OF INITIAL THIAMINE CONCENTRATION ON THE OBSERVED HALF-LIFE OF THE REACTION AT pH 6.37 IN SOLUTIONS DEGRADED AT 96.4°

Initial Thiamine, mg./100 ml.	$t_{1/2}$, hr.
200.0	5.4
100.0	5.5
50.0	5.4
25.0	5.4
12.5	5.5
5.0	5.4
1.0	5.4
0.5	5.5

The previously reported changes of the reaction order might be attributed to changes of pH and also variations of ionic strength of the system. At some pH ranges, the ionic strength apparently has a marked influence on the rate of the reaction.

Dissociation Constants of Thiamine.—As previously stated, the evaluation of the reactions of thiamine requires a detailed knowledge of the species present in the system. For this purpose the dissociation constants were determined at varying temperatures and ionic strength such as to permit species analysis under the conditions of the kinetic runs. From spectrophotometric measurements, based on the method of Flexer (17), pKa_1 was estimated to be 4.0 at 96.4° and pKa_2 (av.) to be 7.8. These values were actually obtained by extrapolation of data determined at lower temperatures from Arrhenius-type plots as shown in Fig. 1 since the instability of the compound precluded deter-

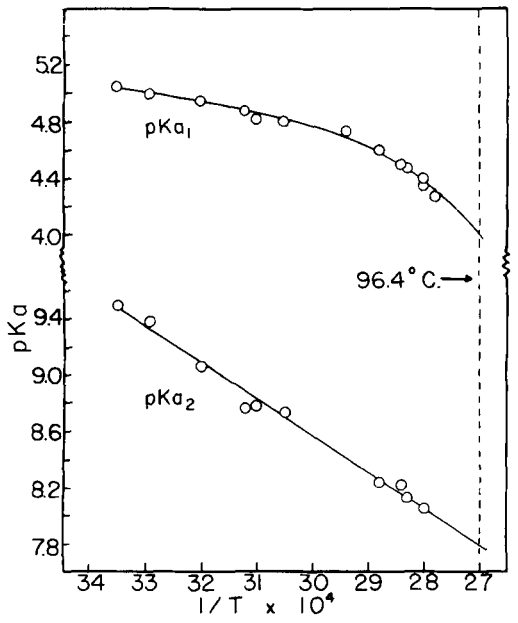


Fig. 1.—Effect of temperature on spectrophotometrically determined pKa_1 and pKa_2 of thiamine at ionic strength 0.5.

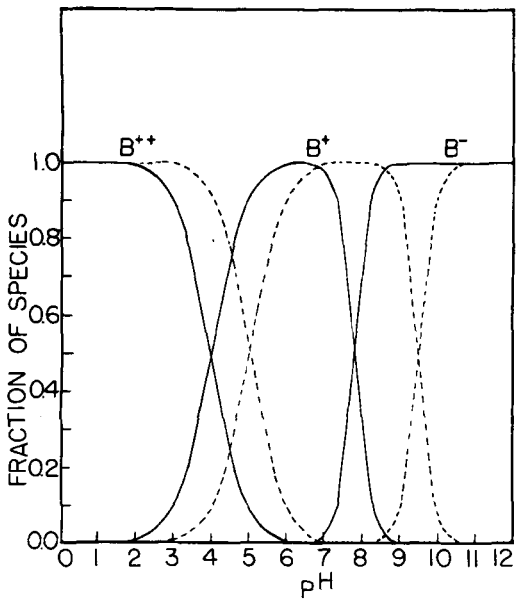


Fig. 2.—Fraction of thiamine species present in solution as functions of pH at 96.4° (—), and 25.0° (---) as calculated from the experimentally determined pKa_1 and pKa_2. B^{++} represents protonated thiamine, B^+ nonprotonated thiamine, and B^- thiol thiamine.

mination at the higher temperature. Figure 2 shows the variations of the thiamine species as a function of pH at 25 and 96.4°. The symbols B^{++} to denote protonated thiamine, B^+ for nonprotonated thiamine, and B^- for the thiol form will be used throughout this presentation.

Identification of the Cleavage Products.—Based on the present findings, and those of others cited, three hydrolytic reactions appear to be responsible for degradation under nonoxidative conditions, the relative importance of each being dependent on the pH of the solution. (*a*) In strongly acid solutions, thiamine apparently degrades by replacement of the primary amino group on the pyrimidine ring by a hydroxyl group to yield oxythiamine. (*b*) The singly protonated and nonprotonated forms which exist in the weakly acid to neutral range seem to undergo simple hydrolytic cleavage at the methylene bridge between the pyrimidine and thiazole groups. (*c*) Under alkaline conditions, where thiamine is present as the thiol form, the end products suggest multiple pathways of degradation. One mechanism resulted in the formation of a pyrimidine diamine and the others resulted in products which have not yet been clearly identified.

To establish the identities of the degradation products over a broad pH range, solutions sealed in ampuls under nitrogen were subjected to hydrolysis at pH values varying from 0.4 to 10.0. The products were separated by a modification of the paper chromatographic technique reported by Lhoest, *et al.* (18). Figure 3 is a composite representation of the chromtographs obtained from the degraded samples along with spots from known compounds. The spots are shown at the average R_f value for each compound and are represented by circles for illustrative purposes.

The results in neutral and acidic media confirmed those of Watanabe (11), in that the end products in this range were 2-methyl-4-amino-5-hydroxymethyl pyrimidine (D) and 4-methyl-5β-hydroxyethyl thiazole (F).

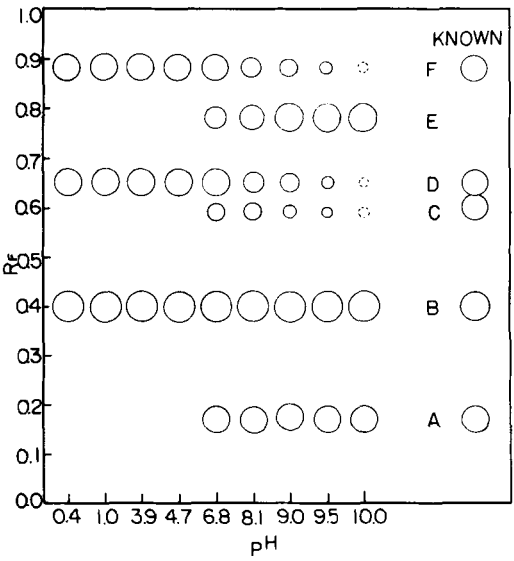
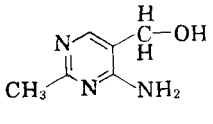
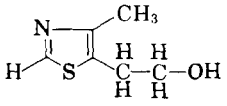


Fig. 3.—Composite representation of paper chromatograms from solutions degraded at 96.4° at various pH's. The spots represent the following compounds: A, 2-methyl-4-amino-5-aminomethyl pyrimidine; B, thiamine; C, thiochrome; D, 2-methyl-4-amino-5-hydroxymethyl pyrimidine; E, unidentified; and F, 4-methyl-5β-hydroxyethyl thiazole. The circles are representative of the size and position of the spots found, not the shape.

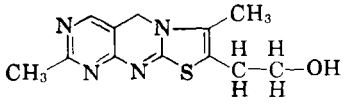


2-Methyl-4-amino-5-hydroxymethyl pyrimidine (D)



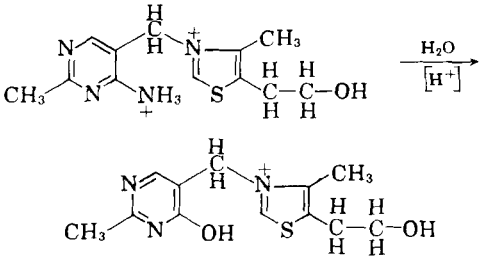
4-Methyl-5β-hydroxyethyl thiazole (F)

These compounds were apparently formed as a result of the hydrolytic cleavage of thiamine at the methylene bridge. Although traces of thiochrome (C) were found on some of the chromatograms, electrofluorometric measurements indicated that the amount formed was negligible. This compound, which is an oxidation product of thiamine may have been produced when the ampuls were opened for removal of sample. At high pH the thiochrome production probably ceased due to the inability of the thiol form of thiamine to yield this compound (15).



Thiochrome

In systems of acid concentration higher than 1 *N* acid, a different route of degradation appeared to become dominant. Rydon (13) found that after refluxing thiamine in 6 *N* hydrochloric acid for 6 hours, a new compound was formed in which the primary amine on the pyrimidine ring was replaced by a hydroxyl group according to the following reaction

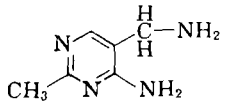


The oxythiamine which was formed did not give the thiochrome reaction.

Chromatography of a solution of thiamine hydrolyzed under Rydon's conditions, indicated that the oxythiamine produced had the same R_f value as thiamine (B) but gave a negative test with ferricyanide reagent. The fact that both thiamine and oxythiamine have the same R_f values in this system may explain why no additional spot was found on chromatographs of solutions degraded at low pH. Only faint traces of the products of the methylene bridge cleavage were found in highly acidic solutions, indicating that the formation of oxythiamine was the main product of the reaction in this range.

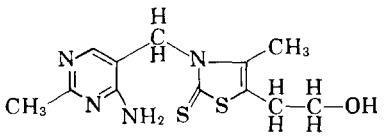
As the pH of the system was increased above 7, the pyrimidine (D) and the thiazole (F) fractions again decreased in intensity. Their decrease was accompanied by the appearance of two new spots, (A) and (E). This progressive change in the nature of the degradation products suggested that the open ring thiol form degraded in a different manner than the closed ring form.

Study of its ultraviolet absorption and paper chromatographic characteristics and reaction to Tollen's reagent and to bromphenol blue-silver nitrate solution suggests that compound (A) is 2-methyl-4-amino-5-aminomethyl pyrimidine, a previously detected degradation product (1, 19).



2-Methyl-4-amino-5-aminomethyl pyrimidine

The nature of the compound giving rise to spot (E) was not clearly established. Other investigators (20, 21) had indicated that at high pH and 100°, thiamine thiothiazolone was formed along with the pyrimidine diamine (A).



Thiamine thiothiazolone

Their conclusions were based on the nature of the ultraviolet absorption spectrum of the degraded solution when adjusted to pH 2.7. It was noted that under these conditions, an absorbance peak was found at 320 mμ which initially increased with time and then decreased, indicating secondary degradation. This absorbance was ascribed to thiamine thiothiazolone as this compound exhibits a marked absorption peak at this wavelength in solutions varying in pH from 2.7 to 9.0. No other thiamine derivative has shown an absorbance peak in this range.

In an effort to identify the compound represented by spot (E), the area was cut out of the paper and the compound eluted with distilled water and the ultraviolet absorption spectrum was determined in solutions at pH 2.8 and 8.5. In the acidic solution a peak was found at 325 and at 243 mμ, the 243 mμ peak being characteristic of the pyrimidine structure. In basic solution, a hypsochromic shift of the spectrum was observed with a new maximum at 305 mμ. Similar results were obtained when samples of the degraded solution were passed through ion exchange columns containing Amberlite IRA-400[1] resin, in the base form, to remove the undegraded thiamine. The eluate adjusted to pH 2.8 and 8.5 exhibited the same spectral characteristics as the samples eluted from the paper. The absorbance at 325 mμ was followed in a kinetic manner, and the results indicated that the compound which was responsible for the observed absorbance appeared to be formed directly from thiamine, based on no observable lag in the time between the initiation of degradation and increase of absorbance. These results were also borne out by following the kinetic procedure by paper chromatographic separations.

The shift of the spectrum of the isolated com-

[1] Rohm and Haas Co.

pound as compared to the stability of known thiamine thiothiazolone possibly casts some doubt on the assumption that the thiothiazolone compound is one of the major degradation products of the reaction under the conditions of this study.

Effect of Ionic Strength on the Reaction Rate.—As might be expected from the various possibilities of thiamine to exist in a charged state, it was found that the rate of the reaction was influenced by ionic strength. The salt effects varied as a function of the thiamine species present and of the mechanism of degradation. It is of pharmaceutical interest, that where the salt effect was operative, an increase of ionic strength resulted in a lower rate of degradation. As pointed out earlier, this effect might also have been a contributing factor to the apparent changes in the order of the reaction found by others (1–3).

Solutions were prepared at pH 2.0, 3.90, 6.40, 6.95, and 10.0 and all degraded at 96.4° except the pH 10.0 samples which were run at 75.0° due to the rapidity of the reaction. The results shown in Fig. 4 only depict the changes found at pH 3.90, 6.40, and 6.95, because at pH 2.0 and 10.0 the reaction was found to be independent of ionic strength. In all cases where changes in rates were observed, it was noted that a plateau existed over which the rates were invariant in respect to ionic strength. For the purpose of accuracy, all subsequent work was conducted at ionic strength 0.5 as this value corresponded to the middle of the plateau at each pH.

To conduct these studies, solutions were prepared at constant thiamine concentration, constant buffer concentration, constant pH, but at varying ionic strength. The ionic strength was controlled by the addition of sodium chloride.

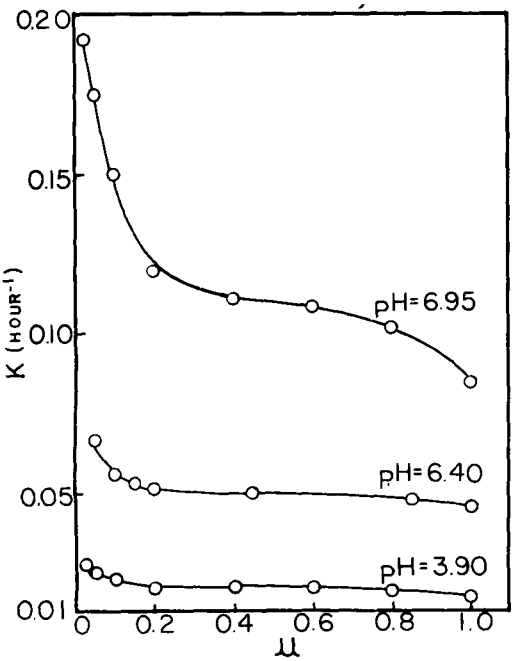


Fig. 4.—Effect of ionic strength on the observed overall rate of hydrolysis at 96.4° at several pH values.

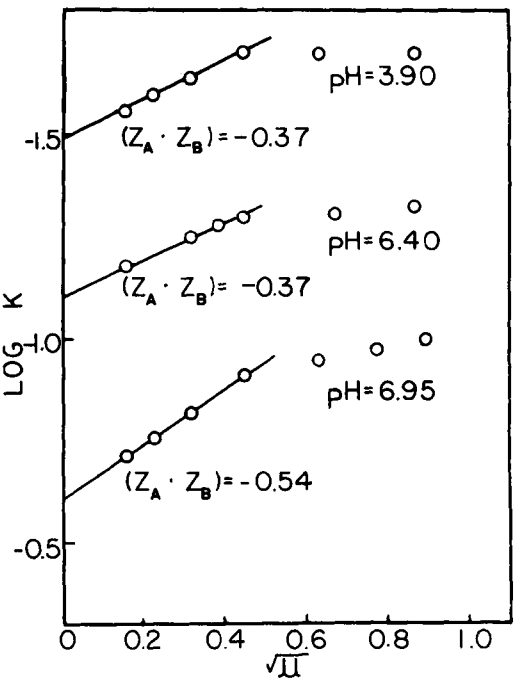


Fig. 5.—Bronsted-Bjerrum-type plot of the logarithm of the observed rate constants against $\sqrt{\mu}$ to estimate the charges on the interacting species at 96.4°.

The data were analyzed by utilization of the Bronsted-Bjerrum equation (22), which relates the primary salt effect on the observed rate to the ionic strength. Figure 5 shows the results of the treatment of the data in this manner. As might be expected, the experimental values deviate rapidly from linearity as this relationship was derived on the assumption of low ionic strength, but the values obtained from the initial slopes were of interest. The finding of nonintegral values for $(z_a \cdot z_b)$ might be due to high ionic strength, the occurrence of more than one reaction at the point of determination, or to a significant contribution by the secondary salt effect. A more complete correlation of these effects will be taken up under the discussions leading to a possible rate equation describing the pH profile of the reaction.

Effect of Buffers as General Acid or General Base Catalysts.—Although earlier studies utilizing phosphate, acetate, and citrate buffers have shown that hydrolysis of thiamine was apparently hastened in their presence, no serious analysis of this effect was made. The findings of the present study indicated that the vitamin was subject to general base catalysis but not to general acid catalysis, the effect being functions of both the buffer species and the forms of thiamine present in the solution. Figures 6–9, representing the results of these studies, are shown as plots of the reciprocal of the half-lives of the reactions *vs.* buffer concentrations under conditions of constant pH. In these studies, solutions were prepared at constant ionic strength, constant pH, and constant thiamine concentration, with only the buffer concentration varying at a given pH.

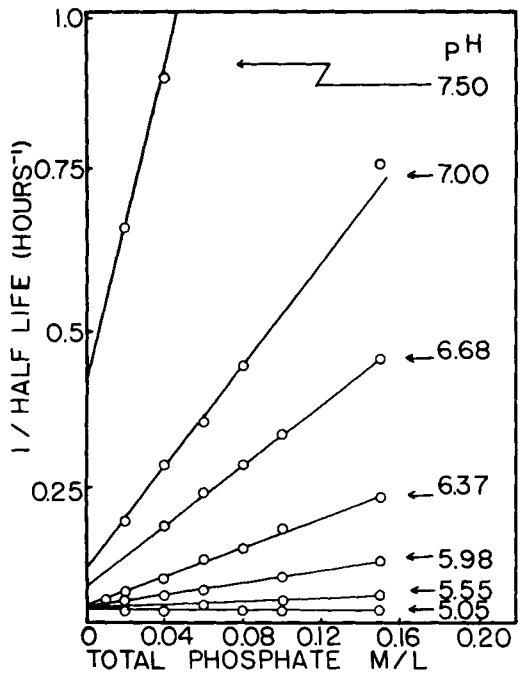


Fig. 6.—Reciprocal of the half-life of thiamine hydrolysis in a phosphate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

This was repeated at a number of pH values within the effective range of the buffers employed.

It was not possible as a part of this investigation to separate clearly the observed total buffer catalysis into the specific contributions of the several general acids and bases present acting on any or several of the thiamine species in solution. If the catalytic activities of these systems are largely limited to their base forms, as apparently indicated by the increasing slopes of the lines in the plots at higher pH, it can be readily shown that

$$[H^+] = Ka \cdot a(1/m) - Ka$$

where Ka is the apparent dissociation constant of the buffer at the temperature of the study; a is a proportionality constant between the slope of the line and the concentration of the conjugate base; m is the slope of the line resulting from the plot of the reciprocal of the half-life against buffer concentration at constant pH. The equation is, as indicated, based on the assumption that only the conjugate base of the buffer is catalytic and that the concentration of the effective substrate is constant. It predicts that a plot of $[H^+]$ against the reciprocal of the slope should yield a straight line with a slope of $Ka \cdot a$ and having an intercept of $-Ka$.

Figure 10 shows the data obtained from the acetate system plotted in accordance with the above equation. The linearity of the experimental points in the higher pH range where essentially only nonprotonated thiamine exists is in conformance with the derived equation. At lower pH, a deviation is noted which indicates that protonated thiamine is perhaps slightly more prone to acetate catalysis. Extrapolation to the intercept yields the dissociation constant of the buffer 1×10^{-5}, which is in good agreement with the experimentally determined value at 96.4°.

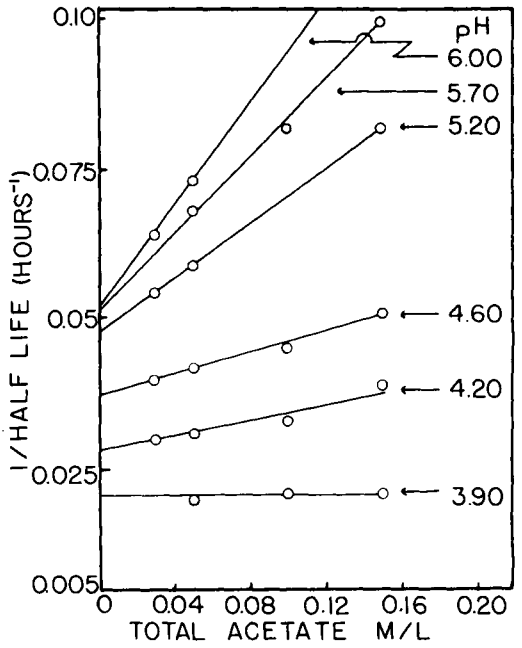


Fig. 7.—Reciprocal of the half-life of thiamine hydrolysis in an acetate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

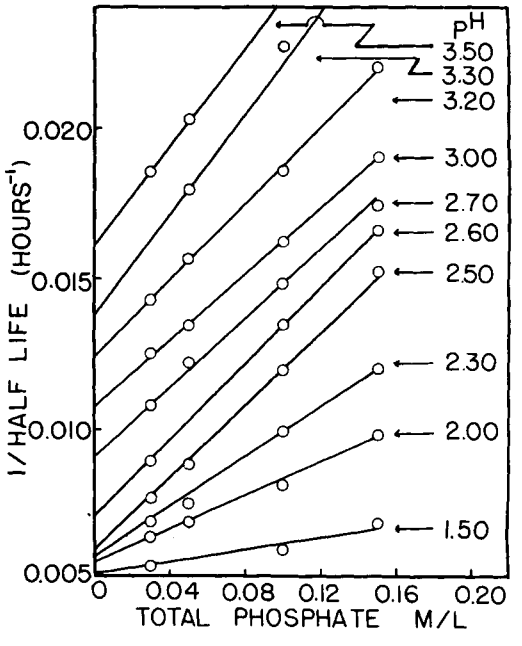


Fig. 8.—Reciprocal of the half-life of thiamins hydrolysis in a phosphate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

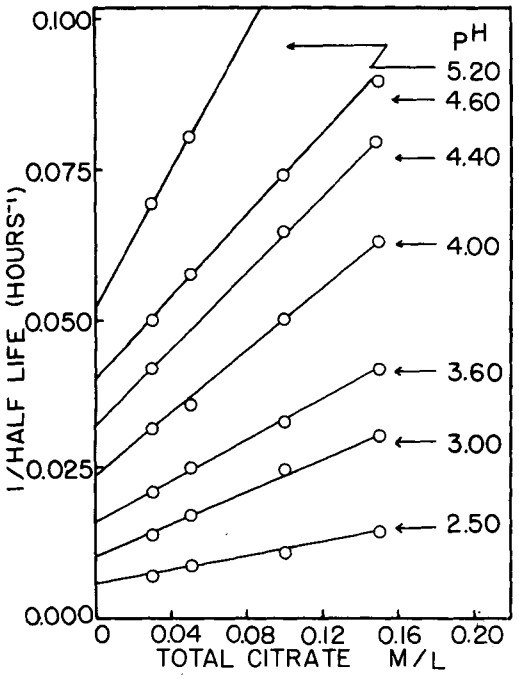


Fig. 9.—Reciprocal of the half-life of thiamine hydrolysis in a citrate system at 96.4°. Effect on the reaction rate of changing buffer concentration at constant pH and constant ionic strength.

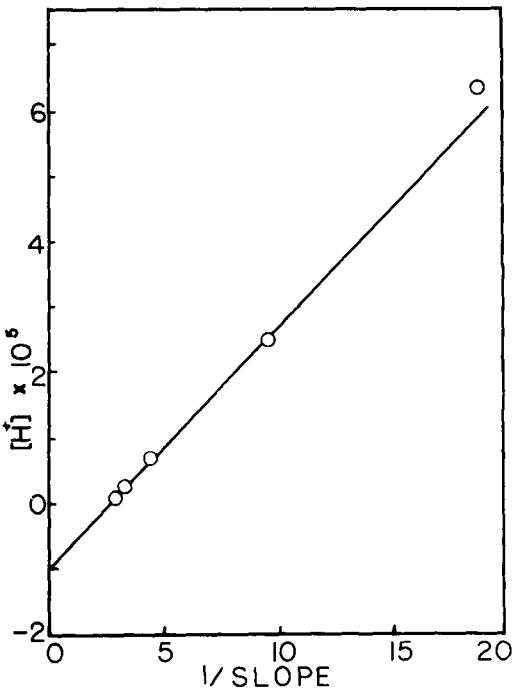


Fig. 10.—Plot of $[H^+]$ *vs.* the reciprocal of the slope of the lines from the acetate system of Fig. 7 in accord with the equation: $[H^+] = Ka\,(a)\,(1/m) - Ka$.

Analysis of the data presented in Fig. 7, depicting the results of degradation in a phosphate buffer system in the pH range 5–7.5, indicates that this reaction may be a function not only of the dibasic phosphate ion, but of the tribasic phosphate ion as well. In this pH range, the nonprotonated thiamine is in equilibrium with the thiol form, whose concentration will increase as the pH is increased. From studies at pH 10, it has been found that the thiol thiamine is not subject to general base catalysis. The essentially zero slope of the line at pH 5.05 indicates that the monobasic phosphate salt is noncatalytic with respect to the nonprotonated thiamine. From these observations it can be suggested that the results found are due to the following reactions

$$B_1 \text{ (nonprotonated)} + HPO_4^{=} \rightarrow \text{products}$$

$$B_1 \text{ (nonprotonated)} + PO_4^{\equiv} \rightarrow \text{products}$$

Mathematical treatment of the data based on the above reactions yields results which are in good agreement with the experimental findings up to pH 7. Above this value there is a negative deviation of the calculated values from the experimental. This deviation may be indicative that the reaction is more complex than anticipated in this region.

Further investigation of the phosphate catalysis was carried out in the pH range 1.5–3.5. The experimental data as shown in Fig. 8 indicate that protonated thiamine is catalyzed by monobasic phosphate ion and not attacked by the undissociated acid. Calculations based on this assumption, together with the previous supposition that the nonprotonated thiamine was not catalyzed by monobasic phosphate, were in good accord in the experimental findings.

The catalytic effects of a citrate system are shown in Fig. 9. From this plot it can be suggested that again the undissociated acid form of the buffer has no catalytic action, but that the buffer anions act as general base catalysts. The interactions are not postulated on a species basis, as this system is too complex to lend itself to this treatment.

Effect of Various Compounds on the Rate of Hydrolysis.—It has previously been reported authoritatively in the literature (2) that a number of compounds such as amino acids, amides, and amines had a marked and surprising effect on the rate of this mode of degradation. A concentration of 0.05 moles per L. of glycine added to a thiamine solution at pH 6.0 apparently produced a threefold decrease in the rate of hydrolysis at 100°. The addition of the same concentration of γ-aminobutyric acid under the same conditions, on the other hand, appeared to increase the rate of the degradation fourfold. Similar effects were found with other compounds and it was noted that in the case of the amino acids, α and β acids tended to stabilize thiamine, whereas those with the amino group further removed from the carboxyl group enhanced degradation. In the cases of the other compounds tested, the effects could not be so clearly related to structural changes.

The magnitude of the effects apparently observed at the low concentrations employed was surprisingly large, in fact so great as to be rationalized on the basis of complex formation between the thiamine and the added agent which might enhance or retard hydrolysis. This type of behavior has been re-

ported by other investigators (23, 24), compounds such as procaine and benzocaine having been stabilized by complexation with a number of xanthine derivatives. Because of the great theoretical significance of this effect, if real, a number of compounds studied earlier were reinvestigated. Table II summarizes the results of present investigations designed to retest this effect.

TABLE II.—EFFECTS OF A SERIES OF COMPOUNDS IN 0.05 *M* CONCENTRATION ON THE OBSERVED HALF-LIFE OF THIAMINE HYDROLYSIS AT pH 6.0 AND 96.4°

Compound Added	$t_{1/2}$, hr.
Control	9.5
Glycine	7.4
Alanine	7.5
Nicotinamide	7.1
Nicotinic acid	7.3
Isonicotinic acid	7.5
Picolinic acid	9.7
Salicylic acid	8.6

In obtaining these results, 0.05 mole per L. of the compound being tested was added to separate thiamine solutions maintained at pH 6.0 by a 0.1 molar phosphate buffer. The ionic strength of the system was maintained at 0.5 by the addition of sodium chloride. Data shown represent means of duplicate runs, reproducibility of the result being of the order of 0.1 hr.

Contrary to the previously published findings, glycine and alanine were found not to exhibit any stabilizing effects on the reaction, but instead showed slight catalytic activity. The other compounds listed, with the exception of picolinic and salicylic acids, exhibited approximately the same slight degree of catalysis. This limited study suggests that the previously reported remarkable changes brought about by the use of some of these compounds may well have been due to other kinetic variables such as ionic strength effects, pH changes, or general base catalysis.

pH-Rate Profile of Thiamine Hydrolysis.—Although the marked influence of hydrogen ion concentration on the rate of the hydrolytic degradation of thiamine was recognized by many early workers (4–8), no previous serious attempts clearly separating the influence of pH from the effects of buffer catalysis seem to have been made. Buffer-free rates at various pH values can, however, be readily picked from Figs. 6–9 since the intercepts of these plots represent the hydrolytic rates at zero buffer concentration. These rates expressed as first-order rate constants are shown in Fig. 11 as a function of pH.

The data shown for pH values above 7.5 were actually run at 75.0° since the hydrolytic rates were too fast at 96.4°, the temperature used for the more acid systems. Actual values obtained at the lower temperature are shown plotted in Fig. 12. These were corrected to the higher temperature by determining the thermal coefficient as shown in Fig. 13, and are represented on the profile by solid circles.

The profile suggested that the overall degradative rate represented a summation of a relatively large number of separate reactions. This is apparent in the analysis of the plot in terms of its observed slope over limited ranges as shown in Table III.

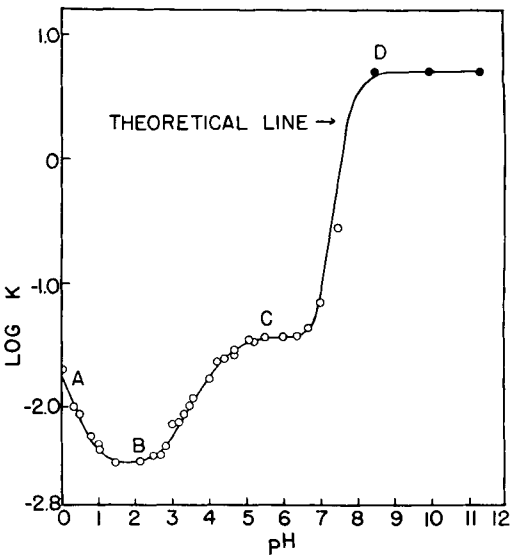


Fig. 11.—pH-rate profile of thiamine hydrolysis at 96.4°. The circles represent the experimental results and the line corresponds to that expected theoretically from the four proposed reactions. The solid circles were calculated from lower temperature studies.

As with all kinetic relationships, a number of different chemical reactions can lead to the same observed experimental dependencies. For the present system a series of four reactions such as

1. protonated thiamine (B^{++}) + [H^+] $\xrightarrow{k_{H^+}}$
2. protonated thiamine (B^{++}) + H_2O $\xrightarrow{k_{H_2O}}$
3. protonated thiamine (B^{++}) + OH^- $\xrightarrow{k_{OH^-}}$
4. thiol thiamine (B^-) + H_2O $\xrightarrow{k'_{H_2O}}$

would be sufficient to fit the experimental points. It is apparent that we can write

$$-dB_T/dt = k_{H^+}[B^{++}][H^+] + k_{H_2O}[B^{++}] + k_{OH^-}[B^{++}][OH^-] + k'_{H_2O}[B^-]$$

where k_{H^+} k_{H_2O} k_{OH^-} and k'_{H_2O} are the specific catalytic constants and B_T represents the total thiamine. By substituting for the thiamine species in terms of their equilibrium concentrations in the manner previously utilized by Higuchi, *et al.* (25), in their studies of procaine hydrolysis, the equation can be rewritten to yield the following

$$k_{obs} = k_{H^+}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right][H^+] + k_{H_2O}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right] + k_{OH^-}\left[1 - \frac{Ka_1[H^+]^2 + Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right] \times \frac{k_w}{[H^+]} + k'_{H_2O}\left[\frac{Ka_1(Ka_2)^2}{[H^+]^3 + Ka_1(Ka_2)^2 + Ka_1[H^+]^2}\right]$$

Ka_1 is the equilibrium constant for the conversion

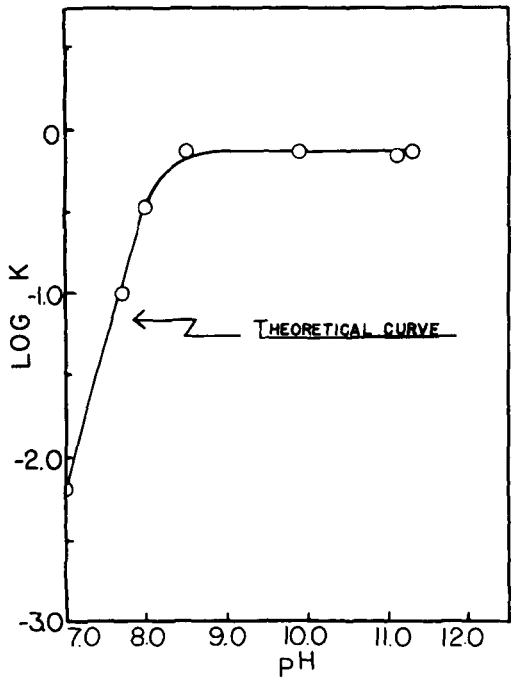


Fig. 12.—Logarithms of experimentally determined overall rate constants for the hydrolysis shown as functions of pH at 75.0°. The solid line corresponds to the theoretical function calculated from the observed rate at pH 9.9 and previously determined pKa values.

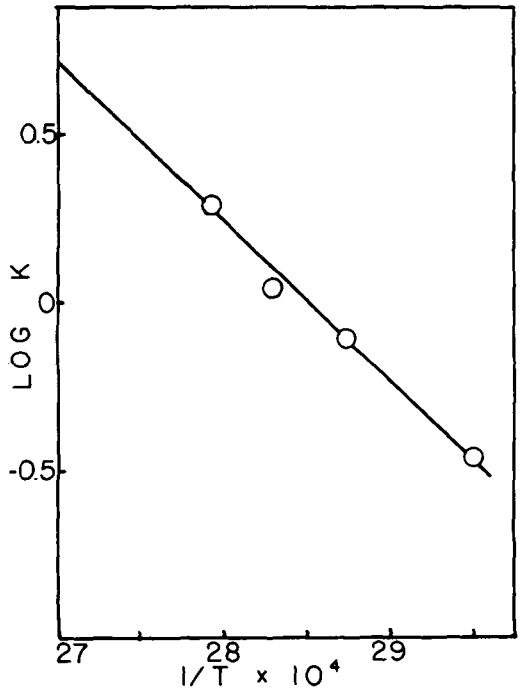


Fig. 13.—Arrhenius-type plot of the logarithm of the observed rate constants against the reciprocal of absolute temperature for the hydrolysis at pH 9.9 under conditions of constant pH. The line is extrapolated to 96.4°.

TABLE III.—APPARENT HYDROXYL ION DEPENDENCY OF THE HYDROLYTIC REACTION

pH Range	Apparent Slope
0–1	−1
1.5–2.5	0
3–4	0.4
5–6	0
7–7.5	2
9–11	0

of protonated thiamine to the nonprotonated form; Ka_2 is the average equilibrium constant for the conversion of the nonprotonated form to thiol thiamine.

It is evident that k'_{H_2O} and k_{H^+} can be readily evaluated from the two extremities of the profile. The remaining two rate constants can be easily calculated from any two points in the central part of the profile and from the two Ka values previously determined. When the constants thus obtained were substituted into the expanded equation, the functions shown as solid lines on Figs. 11 and 12 resulted.

The good agreement of the experimental data and the calculated theoretical profile does not unambiguously establish the nature of the interactions along all segments of the curve. The major reaction along the D portion of the curve on Fig. 11 could be formulated on the basis of nonprotonated thiamine being attacked by two hydroxyl ions or by the spontaneous cleavage of thiol thiamine by water. The choice of the latter interaction, reaction 4 above, as largely representing the D section of the curve is based on the absence of a primary salt effect and general base catalysis in this region; whereas in the alternative case, both of these effects would be expected.

Similar equivalences could be set forth for the dominant reaction along the C segment. Here, the observed effects could be due to protonated thiamine reacting with hydroxyl ion or due to water cleavage of the nonprotonated thiamine; these are kinetically indistinguishable. Again the choice as the most probable mechanism must be made on the basis of other supportive evidence. The negative dependency on ionic strength, as indicated in Fig. 5, points to the reaction of negatively and positively charged groups. The value of −0.37 for the product of the charges of the interacting species does not agree with the −2 predicted by the Bronsted-Bjerrum theory, but this discrepancy might be attributed to the high ionic strength of the experimental solutions as compared with the limitations of the equation. Further, the postulation of a cleavage of the nonprotonated thiamine by water alone would also indicate that B^+ reacted faster with water than B^{++}. This would not be in accord with the results found with buffered systems, where it was found that although the monobasic phosphate ion did not appear to catalyze the degradation of B^+, it was active in respect to B^{++}. In general, it appears that the protonated thiamine is more susceptible to nucleophilic attack than the nonprotonated species. Hence it would appear probable that the main reaction in the C segment can be attributed to B^{++} reacting with OH^-, reaction 3 above.

The failure to find any salt effect at pH 2.0 tends to support the suggestion that the most important

reaction in the B region is reaction 2, cleavage of B^{++} by water. The salt effects observed at pH 3.90 are in agreement with the proposed reactions, as in this region the observed effects are a composite of reactions 2 and 3.

In the strongly acid region designated by the letter A, the reaction is first order in respect to hydrogen ion. From the work of Rydon (13) and our own studies, it appears that the mode of degradation changes from the methylene bridge cleavage associated with reactions 2 and 3, to the hydrolysis of the 4-amino group on the pyrimidine ring. They night be postulated to involve ring protonation with subsequent attack by water.

Although it would appear from the above discussion that the series of four reactions adequately fit the data at hand, the possibility and perhaps the probability that the true situation may involve one or more substitute reactions is acknowledged. Kinetic analyses can readily eliminate many proposed reaction mechanisms, but they cannot establish the correct ones with absolute certainty.

Determination of the Apparent Activation Energies of the Hydrolytic Reactions.—The apparent activation energies of the reactions were determined by measurements of the observed rate constants at varying temperatures under conditions of constant pH. These determinations were made on solutions at pH 0.05, 1.70, 6.37, and 9.90. These points were picked because at each, only one term of the proposed rate equation for the pH dependency of the reaction was significant, therefore it was possible to estimate the temperature dependence of the individual reactions responsible for the profile. At pH 6.37, where buffers were employed, the rates were those obtained by extrapolation to zero buffer concentration.

The results shown in Fig. 13 were obtained from studies at pH 9.90 and changes at the other pH values followed similar linear relationships when log k was plotted against the reciprocal of the absolute temperature. The linearity of the points over the temperature range indicated the adherence of the observed reactions to the Arrhenius equation and allowed calculation of the apparent activation energies from the slopes of the lines. The results are summarized in Table IV.

These energy values appear to be within the expected magnitude based on the stability of the vitamin as found in our studies and by others. These high activation energies are of practical significance in the preparation of sterile thiamine solutions. Near 100°, the rate of the degradation increases by a factor of approximately 2.5 for every 10° rise in temperature. This change in the rate may be important when determining the most suitable conditions for sterilization.

TABLE IV.—APPARENT ACTIVATION ENERGIES FOR HYDROLYSIS DETERMINED UNDER SEVERAL CONSTANT pH CONDITIONS

Corresponding Reaction	pH of Experiment	E_a, Kcal./mole
1	0.05	24
2	1.70	29
3	6.37	25
4	9.90	21

EXPERIMENTAL

Reagents.—All chemicals used in the preparation of the buffers and for the preparation of the kinetic solutions were of analytical reagent grade except for the special compounds listed below: Thiamine chloride hydrochloride was obtained by double recrystallization of U. S. P. grade material from 95% ethanol and dried under vacuum at room temperature, m.p. 247–248°. 2-Methyl-4-amino-5-aminomethyl pyrimidine dihydrochloride was prepared by the acid degradation of thiamine disulfide according to the method of Zima and Williams (19), m.p. 264°. 2-Methyl-4-amino-5-hydroxymethyl pyrimidine hydrochloride was isolated from the hydrolysis of thiamine according to the method of Watanabe (1), m.p. 219°. 4-Methyl-5-hydroxyethyl thiazole hydrochloride was also isolated from thiamine hydrolysis in the manner described by Watanabe (1), m.p. 96°. Oxythiamine hydrochloride was prepared according to the method of Rydon (13), m.p. 193–194°.

Analytical Procedure.—Residual thiamine concentrations were determined by the thiochrome method of U. S. P. XVI (26). All compounds used in the kinetic studies were tested for interference with the analytical method.

Kinetic Procedure.—All solutions for kinetic studies were prepared with glass-distilled water to minimize metal catalyzed oxidation. Prior to the addition of the thiamine to the buffers, the solutions were heated to expel oxygen and cooled under an atmosphere of nitrogen. After the addition of the thiamine, the solutions were filled into ampuls and again flushed with nitrogen before sealing. The samples were then placed in a constant temperature bath which was maintained at the desired temperature, ±0.05°. After allowing 15 minutes for temperature equilibration, the zero-hour samples were removed and further samples were withdrawn at time intervals suitable to the nature of the system. The reaction was quenched by immersion of the samples in ice water.

Control of pH.—In all cases except where citrate buffers were used, the solutions were prepared by precalculation of the buffer concentrations utilizing experimentally determined apparent pKa values of the buffers. The pKa values were determined from pH measurements on solutions containing equal concentrations of the buffer acid and salt at the ionic strength desired. These determinations were made at various temperatures and the results extrapolated to 96.4°. In all cases, the ionic strength was adjusted by the addition of sodium chloride.

In the case of the citrate system, due to its complexity, it was not feasible to precalculate the solutions, therefore the literature pKa values were used to calculate ionic strength and the pH of the solutions were adjusted by the addition of base.

Determination of Thiamine Dissociation Constants.—The acid dissociation constants of thiamine were determined spectrophotometrically using the method of Flexer, *et al.* (17), which was modified by use of circulating apparatus for photometric titrations as described by Rehm, *et al.* (27). This modification allowed titration of the sample to a desired pH, and after recording the spectrum, titrating further without the necessity of preparing separate solutions. The method is based on the determina-

tion of the absorbance of a compound at a pH where it is completely unionized and then measuring the absorbance of the same concentration at a pH where it is completely ionized and at some intermediate point. The apparent pKa_1 was calculated from the following equation

$$pKa = pH - \log A_{B^{++}} - A/(A - A_{B^{+}})$$

where $A_{B^{++}}$ is the absorbance of the protonated thiamine, $A_{B^{+}}$ is the absorbance of the nonprotonated thiamine, and A the absorbance of a solution containing a mixture of the two species. The absorbance was measured at 255 mμ using a Cary recording spectrophotometer. This wavelength was used as it most readily reflected the changes of the species in the solution.

Although the above procedure was suitable for the determination of pKa_1, which is a simple one-step dissociation, modifications in the equation were required for the estimation of the second constant. As described in the earlier sections, the second dissociation constant is essentially an average value for a two-step mechanism, therefore a new model was used in the calculation. The model used was that of a dibasic acid in which the first and second dissociation constants were equal, only allowing measurement of the initial and final forms. Application of this relationship to the thiamine system, the equation derived was as follows

$$pKa_2\ (av.) = pH - {}^1/_2 \log A_{B^{+}} - A/(A - A_{B^{-}})$$

where $A_{B^{-}}$ is the absorbance of the solution when all of the thiamine is in the thiol form. The other symbols are as used above.

The thiamine concentration was maintained at 4 mg./100 ml. throughout the titrations and the ionic strength maintained at 0.5. The change of ionic strength during the titration was negligible due to the swamping effect of the added electrolyte. Solutions were allowed to equilibrate at least 15 minutes after each change of pH before recording the spectrum.

Chromatographic Separation of Degradation Products.—Solutions of thiamine in 1% concentration were prepared at various pH's employing the same methods as used with the kinetic samples, and degraded at 96.4° for a time equivalent to two half-lives of the reaction. Since the observed R_f value of a compound is dependent on the form in which it exists in a system, all solutions were readjusted to pH 3.5 prior to spotting on the paper to maintain constancy of species. The samples were chromatographed using the method described by Lhoest, *et al.* (18), with several modifications. Whatman No. 4 chromatography paper used by the previous workers and sample size was reduced from 0.1 ml. to 0.01 ml. to yield better definition of the spots. Development of the spots was carried out in the same manner as that of earlier workers, except that known degradation products were run along with the degraded solutions. The inclusion of the known compounds allowed identification of most of the spots found from the degraded solutions.

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Analysis of Thymol and Eugenol Employing Ion Exchange Resins

By FLOYD A. WAGNER and MURIEL C. VINCENT

A quantitative analysis for thymol, eugenol, and clove oil has been developed by means of a spectrophotometric-anion exchange procedure. Suitable reagents which will produce colored compounds with small amounts of the phenolic substances have been selected. A comparison with the official assay for clove oil has been made. The ion exchange method indicated a full recovery of the phenols present in the oil and could be conducted in a much shorter period of time than the official method.

THE QUANTITATIVE determinations for volatile oils and their constituents are numerous, and in the case of the oils the assays frequently depend on a particular group of chemical substances rather than a specific compound. In this respect, then, it would be quite desirable to have a procedure capable of rapidly, accurately, and conveniently assaying an aqueous or nonaqueous mixture of such products for the major constituent only.

The presently accepted assay procedure for thyme oil (1) and clove oil (2) for their respective principal constituents, thymol and eugenol, have several disadvantages. These are primarily the nonspecificity of the alkali hydroxide to eugenol and thymol because of its reaction to phenolic compounds in general, inexact measurements of volumes, and inaccurate readings of the meniscus (3).

It was the purpose of this study to develop a method which would rapidly and accurately remove eugenol or thymol from a solution by means of an ion exchange resin.

The application of chromatographic techniques in the analysis of volatile oils has been somewhat limited. However, gas chromatographic analysis has been employed by Naves (4) for thyme oils and by Naves and Odermatt (5) and Stahl and Trennheuser (6) for eugenol. Bielenberg and Fischer (7) have reported the adsorption of thymol and other monohydroxy benzenes on alumina columns. Frydman (8) and coworkers used silicic acid-starch paste-covered glass plates to achieve the separation of thymol and other compounds of volatile oils.

EXPERIMENTAL

Experiments were first undertaken to find reagents which would produce soluble colored compounds with various known concentrations of thymol and eugenol. These solutions were observed at different wavelengths of light using a spectrophotometer to obtain the characteristic curves, and standard curves were then established. Subsequently, samples of thymol, eugenol, and clove oil were passed through a weakly basic ion exchange resin, and the eluates were treated with the reagent and observed at the required wavelength.

Reference to the standard curve showed that quantitative recoveries were obtained.

Color Reagents and Standard Curves.—Alcoholic thymol solutions of 0.1 *M*, 0.01 *M*, and 0.001 *M* concentrations were treated in the following manner with Hammarsten-Rolbert reagent (9). One milliliter of solution to be tested was placed in a cell and 5 ml. each of glacial acetic acid and concentrated sulfuric acid were added to produce the characteristic red-violet color. The solutions were allowed to stand 5 minutes to permit maximum color development before viewing in the Colemen spectrophotometer between wavelengths of 400 and 650 mμ (Fig. 1). The standard curve was prepared from the data, using transmittance at 520 mμ (Fig. 2).

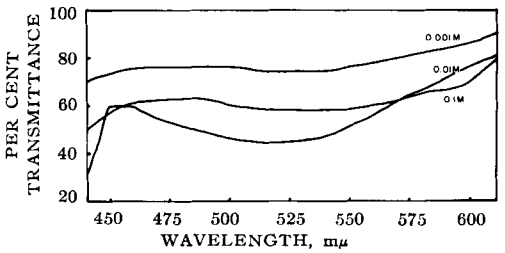


Fig. 1.—Concentration-transmittance curves for thymol.

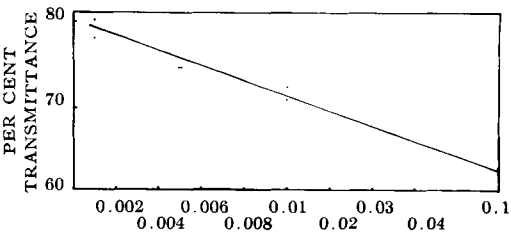


Fig. 2.—Standard curve for thymol, 520 mμ.

Received April 28, 1961, from North Dakota State University, College of Pharmacy, Fargo.
Accepted for publication July 21, 1961.
Abstracted in part from a thesis presented by Floyd A. Wagner in partial fulfillment of the requirements for the degree of Master of Science.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

TABLE I.—ANALYSIS OF PHENOLS AND PHENOL-CONTAINING OILS

Phenol or Volatile Oil	*T*, %	Concentration, *M*	Phenols,[a] %	Phenols Recovered,[b] %
Thymol	73.8	0.0067	.....	100.0 ± 2.99 (11)[c]
Eugenol	23.1	0.0251	.....	100.0 ± 0.80 (3)
Clove oil	21.8	0.0289	87.9 ± 2.0 (3)	98.8 ± 2.10 (3)

[a] Calculated by official assay. [b] Analyzed by ion exchange-spectrophotometric method. [c] Number of determinations.

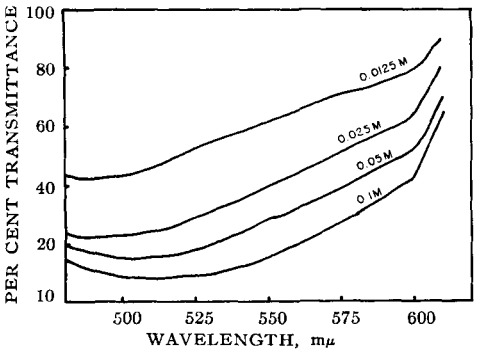


Fig. 3.—Concentration-transmittance curves for eugenol.

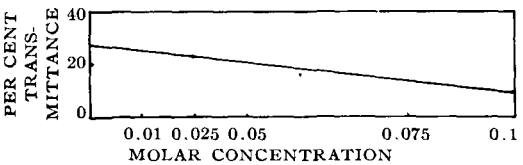


Fig. 4.—Standard curve for eugenol, 500 mμ.

Solutions of eugenol in dimethylformamide were prepared in concentrations of 0.1 *M*, 0.05 *M*, 0.025 *M*, and 0.0125 *M* and treated with Ehrlich's diazo reagent (10). In performing the test, 3 ml. of sulfanilic acid solution was added to 3 ml. of sodium nitrite solution previously placed in a cell; to this solution was added 0.1 ml. of eugenol sample and finally 3 ml. of sodium bicarbonate solution. The resulting yellow-brown solution was observed in the spectrophotometer after 15 minutes and the transmittance of light recorded between 400 and 650 mμ (Fig. 3). The standard curve was based on the transmittance of light at 500 mμ (Fig. 4).

Ion Exchange Procedure.—The weakly basic anion exchange resin, Amberlite IR-45, was employed. Ten-gram samples of the air dried resin were moistened with water, transferred to columns (22 × 3 cm.), and regenerated with 50 ml. of 4% sodium carbonate solution. After regeneration and rinsing of the columns, the distilled water was removed from the columns by washing with 30 ml. of alcohol or dimethylformamide for thymol and eugenol, respectively.

The sample to be analyzed was then added to the columns by means of a graduated 1-ml. pipet to provide 50 to 100 mg. of thymol or eugenol. All columns were washed with alcohol or dimethylformamide at a rate of 1 ml. per minute until 100 ml. of eluate was collected in a volumetric flask. After thorough mixing of the eluate, the color developing agent was added as previously described. Blank determinations were made with all samples.

Table I shows the data obtained when the phenols and clove oil were analyzed in this manner. Comparative official assays were performed on clove oil.

SUMMARY

A quantitative analysis for thymol, eugenol, and clove oil has been developed by means of an anion exchange-spectrophotometric procedure.

Suitable reagents which will produce colored compounds with small amounts of the phenolic substances have been selected. Ehrlich's reagent, which produces an azo dye with eugenol, is very sensitive and will give the characteristic yellow-brown color with eugenol in quantities as minute as 0.2 mcg. The Hammarsten-Rolbert reagent will give the characteristic red-violet color of thymosulfonic acid with amounts as low as 10 mcg. of thymol.

A comparison with the official assay for clove oil has been made. The method indicated a full recovery of the phenols present in the oil and could be conducted in approximately 3.5 hours, which is considerably less than the 18 hours required for the official assay.

Attempts to perform a similar assay on thyme oil gave consistently high results, which indicated that the reagent evidently reacted with other components of the oil. Thus, more specific color reagents for thymol will have to be developed or more selective ion exchange resins employed. Such improvements are presently being investigated.

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Pharmacognosy of Indian Substitutes of Male Fern II

Dryopteris barbigera and *Dryopteris cochleata*

By P. N. MEHRA and T. C. MITTAL

A macroscopic and microscopic description of the rhizome and leaf bases of *Dryopteris barbigera* (Moore) O. Ktze. and *D. cochleata* (Don) C. Chr. is presented. Both the species compare very favorably in oleoresinous content and 'crude filicin' with the official male fern. The two species, described in this paper are differentiated from each other on the basis of the shape and size of the rhizome, structure of the ramenta present on the rhizomes, the presence and absence of dark sclerotic nests in the ground tissue, the number of cauline vascular bundles, and the shape and size of the stalk of internal secretory unicellular glandular hairs.

EXTRACTUM FILICIS has been extensively used as a teniacide for the last two centuries or so (1). Since the drug has been included in various pharmacopeias and also the International Pharmacopoeia, its importance cannot be overemphasized. Considerable quantities of the expensive male fern extract are imported into India for medicinal purposes, since both the official species, *Dryopteris filix-mas* (L.) Schott and *D. marginalis* (L.) Asa Gray are not met with in India. Search for an Indian substitute for this important drug has shown that there are some excellent substitutes that grow wild in the Himalayas, some abundantly enough to be commercially exploited (2, 3). The detailed pharmacognosy of two of them has already been published (4), while that of *D. barbigera* (Moore) O. Ktze. and *D. cochleata* (Don) C. Chr. is described in this communication. The oleoresin content of both the species varies from 7 to 9% and the "crude filicin" in *D. barbigera* is approximately 2.2% and in *D. cochleata* up to 1.8% (2).

The purpose of the study is to report the macroscopic and microscopic characteristics of these two species in order to establish satisfactorily their identity in commerce. The description of the plant has been provided to facilitate its collection, as no such appropriate description is available in previous literature on Indian ferns (5–11).

MATERIAL AND METHODS

The material for *Dryopteris barbigera* was gathered from alpine meadows above Khillanmarg (Kashmir) and en route to Rohtang pass (Kulu Hills), between altitudes of 10,000–13,000 feet, where it grows in fair abundance. The sample of *D. cochleata* was collected from Raiwala forests, Dehradun (Western Himalayas), and Majitar bridge (Eastern Himalayas), between altitudes of 1,500–3,000 feet where it occurs gregariously. The technique employed was the same as described in an earlier communication (4).

Received June 30, 1960, from the Department of Pharmacognosy, Panjab University, Chandigarh (India).
Accepted for publication May 23, 1961.

INVESTIGATIONS

***Dryopteris barbigera* (Moore) O. Ktze.**

Plant.—The rhizome is thick, ascending, sometimes branched, copiously covered with chaffy, chestnut brown, large ramenta. The stipes are tufted, 15–30 cm. long, rachis as well as the stipes are densely covered with large, bright, rufo-brown, imbricate ramenta, and soft silky hairs; lamina 60–90 cm. long, ovate-lanceolate (Fig. 1, part 1). Pinnae are close, up to 20 pairs or more, 8–15 cm. long, 2.5–4 cm. broad, gradually reduced below, linear-lanceolate. Pinnules are usually distinct, ovate-oblong, double serrate, or deeply lobatoincised and each lobe again provided with copious aristate teeth, the edge often incurved; texture herbaceous, both the surfaces copiously fibrillose-scaly, more so the lower surface; midrib fibrillose-scaly; sori in two rows close to the midrib of the pinnules; indusium small, entire, pale colored, of thick texture with small and shallow sinus.

Macroscopy of the Rhizome.—Rhizome may be branched or unbranched, of variable shapes, ovoid, oblong, or elongated (Fig. 1, part 2), measuring up to 20 cm. long and up to 6 cm. or more thick at the crown. Externally reddish-brown, entirely covered by thick, stout, persistent, curved, or flattened, or angular frond bases which are up to 15 mm. thick and somewhat wrinkled. Long shreds of hypodermal sclerenchyma are observed attached to the frond base. The entire rhizome and stipe bases are densely clothed in rufus-castaneous, broadly lanceolate, hair-pointed scales which are 2–4 cm. in length. The rhizome breaks with hard and uneven fracture. Transversely cut surface, which in fresh drug is markedly pale green and in dried drug is brown, reveals 6–9 whitish patches of cauline meristeles arranged in a diffused circle and 4–5 leaf-traces, each composed of 10–13 smaller vascular strands, arranged in the form of closed 'C' at angular regions. Odor slight, taste at first sweetish, later becoming acrid and nauseous.

The ramenta are ovate to lanceolate with acuminate apex, each measuring 2–4 cm. or more in length,

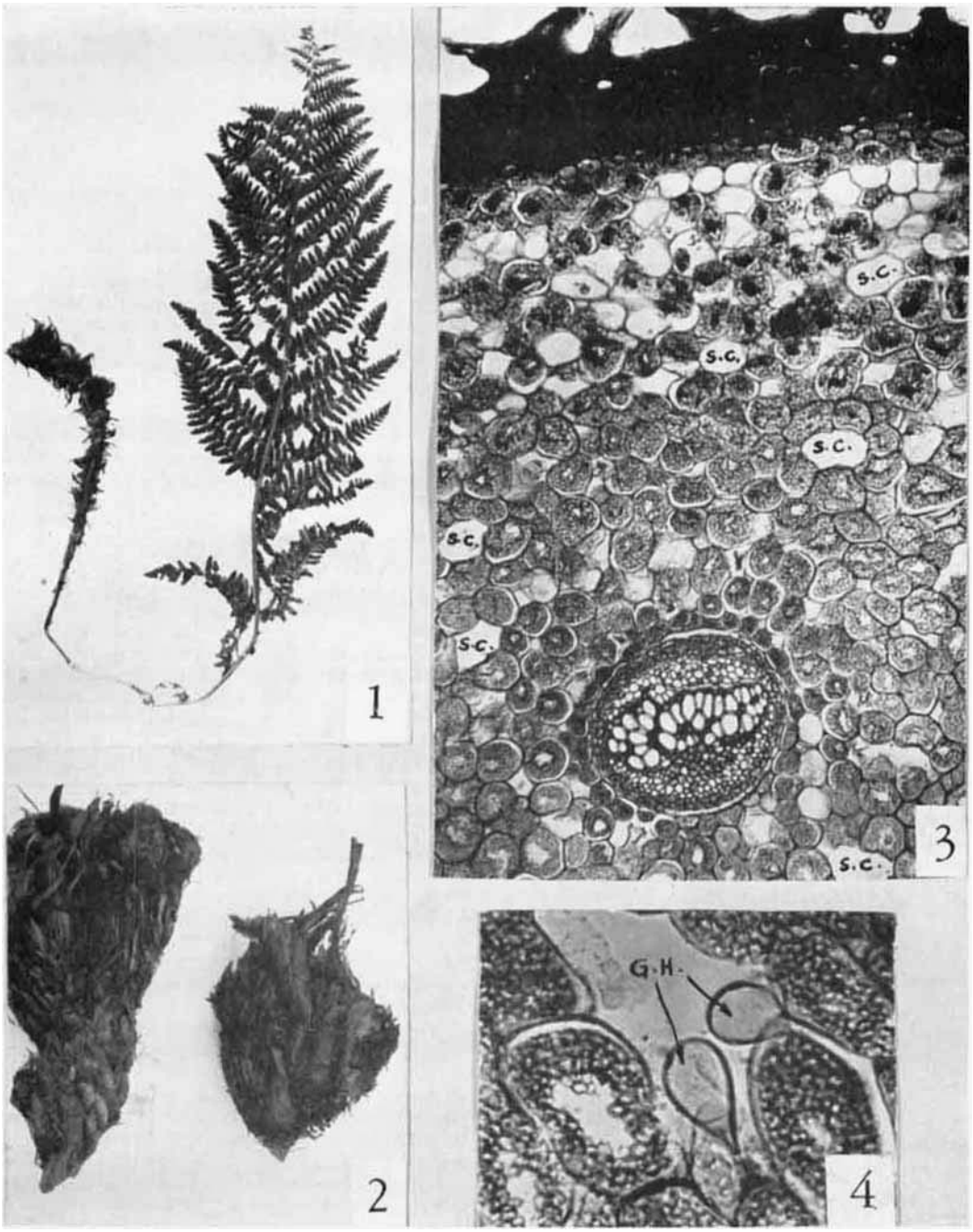


Fig. 1.—*Dryopteris barbigera* frond, × 1/5. 2, Rhizome, × 1/3. 3, Sector of T.S. of the rhizome magnified × 200; S.C., schizogenous cavity. 4, L.S. of the rhizome showing schizogenous cavity containing unicellular secretory glandular hairs, × 250; G.H., glandular hairs.

and 3–8 mm. in breadth. Each ramentum is composed of thin-walled, elongated cells, each measuring 500–1200 × 20–30 μ. At places the marginal cells form peg-like projections, each consisting of two parallel and contiguous cells. No glandular hairs are observed on the margin or the surface of the ramenta.

Histology of the Rhizome.—A transverse section of the rhizome from the periphery to the center is shown in Fig. 1, part 3. The epidermis is composed of a single layer of cells which vary in shape from oblong to rectangular. These cells are brownish in color and contain occasional tannin-resin masses. Some of the cells bear unicellular pyriform glandular hairs, and a few others the ramenta cut variously. Since the rhizome is profusely scaly a large number of cut ramenta are observed in a cross section.

The hypodermis is differentiated into two zones,

an outer one of 2–3 layers of cellulosic fibers, and an inner band of 5–8 layers of thick-walled lignified fibers. The cells of the latter appear yellowish to orange in unstained preparations due to the deposition of tannin-like material on the walls. In T.S. the cells of the inner zone appear polygonal and are more or less isodiametric, but in maceration they are 10–20 times as long as broad and possess flat or somewhat tapering ends (Fig. 2*a*). This region is compact and lacks intercellular spaces.

The cortex is composed of several layers of rather thick-walled parenchymatous cells, often filled with simple starch grains. Besides starch, the cells, particularly next to the hypodermis and around the meristeles, contain spherical masses or granule aggregates of tannins. The walls of such cells are cellulosic in nature while the remaining cells possess suberized walls. Some of the cells possess simple pits on their walls and such cells are devoid of inclusions. The cortical cells in maceration appear either isodiametric or slightly elongated (Fig. 2*c*), each measuring 70–90 × 40–90 μ. In T.S. they number about 400/mm.^{2}.

The cortex shows numerous small intercellular air spaces and large schizogenous cavities. Within the latter project characteristic unicellular glandular hairs (Fig. 1, part 4), each surrounded by greenish-yellow oily secretion. The glands are 60–100 μ long of which 20–25 μ is the stalk. The head is oval or pyriform in shape and measures 35–56 μ in diameter. The schizogenous cavities in the region of their maximum distribution number about 45/mm.^{2}, as observed in T.S. In longitudinal section, the cavities measure 160–350 × 60–160 μ and each contains 2–3 glands.

Sclerotic nests are absent both in the cortex and the pith. Pith cells are identical with the cortical cells.

Within the ground tissue is present a diffused ring of 6–9 large cauline meristeles. Numerous smaller strands arranged in the form of closed 'C' constituting leaf traces are present at angular regions of the rhizome. Occasionally root traces are also observed, passing out of the cortex.

Meristele.—The cauline meristeles and the leaf trace bundles are oblong to elliptical in outline and amphicribral in nature. The leaf trace bundles are relatively much smaller and in longitudinal sections are observed to arise from the margin of the leaf gaps.

Each meristele shows a single-layered endodermis of rectangular cells, the walls of which are suberized and the radial ones possess feebly developed Casparian thickenings. This is followed by 1–3 layers of thin-walled, nonlignified parenchymatous pericyclic cells, filled with starch grains and amorphous tannin deposits. In cross section the cells appear polygonal to oblong in shape. Phloem is many layered and encircles the central xylem on all sides except at two lateral ends where the pericycle directly abuts on the xylem. It consists of sieve tubes and phloem parenchyma. The cells of the phloem parenchyma are filled with tannin deposits. Xylem is central in position and consists of tracheids and xylem parenchyma. The tracheids in cross section have typical polygonal outline. In maceration they appear elongated and the metaxylem elements show scalariform thickenings. The bundle, according to its size, may have 2–3 small protoxylem groups consisting of small annular or spiral tracheids; frequently one is found at each end of the xylem mass.

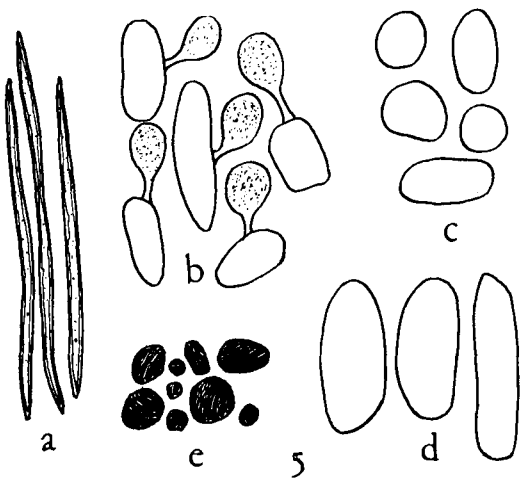


Fig. 2.—*Dryopteris barbigera*, tissue macerations. *a*, Hypodermal fibers, × 160; *b*, gland bearing cortical cells, × 160; *c*, ordinary cortical cells of the rhizome, × 160; *d*, ordinary cortical cells of the stipe base, × 160; *e*, starch grains, × 700.

Histology of the Stipe.—In T.S. it is somewhat oval in outline. The hypodermis is well developed and formed of lignified fibers. Unlike the rhizome it is not differentiated into two zones. The cells of the cortex are 2–3 times larger than those of the rhizome (Fig. 2*d*), and are predominantly oblong or rectangular. The few layers that lie next to the hypodermis contain tannoid aggregates, but such contents are missing from other cells. The cortical cells number about 250/mm.^{2} in contrast to nearly 400 in the rhizome. The number of schizogenous cavities per unit area is almost the same as in the rhizome. The number of vascular strands varies from 10–14. They are arranged in the form of closed 'C', and the two adaxial ones are larger with the xylem notched internally.

Dryopteris cochleata (Don) C. Chr.

Plant.—Rhizome is ascending, and clothed in golden yellow, ovate-lanceolate, or linear-subulate ramenta. Fronds are strongly dimorphic (Fig. 3, part 6); the stipe is up to 40 cm. long, comparatively much longer and thicker in fertile fronds, tufted; main rachis glabrous; lamina up to 40 cm. long, deltoid or ovate-oblong with slightly cordate or rounded base. Pinnae are up to 10 pairs, basal pinnae not any longer than the ones next above, not much basiscopically produced, ovate-lanceolate, acuminate, the sterile basal ones are subsessile or sessile, up to 13 cm. long and 4 cm. broad, fertile ones distinctly though shortly petiolate, up to 8 cm. long and 2 cm. broad, the lower ones ascending, others patent; the rachis deeply canaliculated above, the underside of the rachis and rachises of fertile fronds fibrillose but of sterile fronds is setose hairy; sterile pinnules more or less adnate to a winged costule, oblong-obtuse, base truncate, serrate; fertile pinnules subsessile with serrate margin; texture subcoriaceous, green or pale green, veins inconspicuous; sori large, well below the middle of upper veinlet

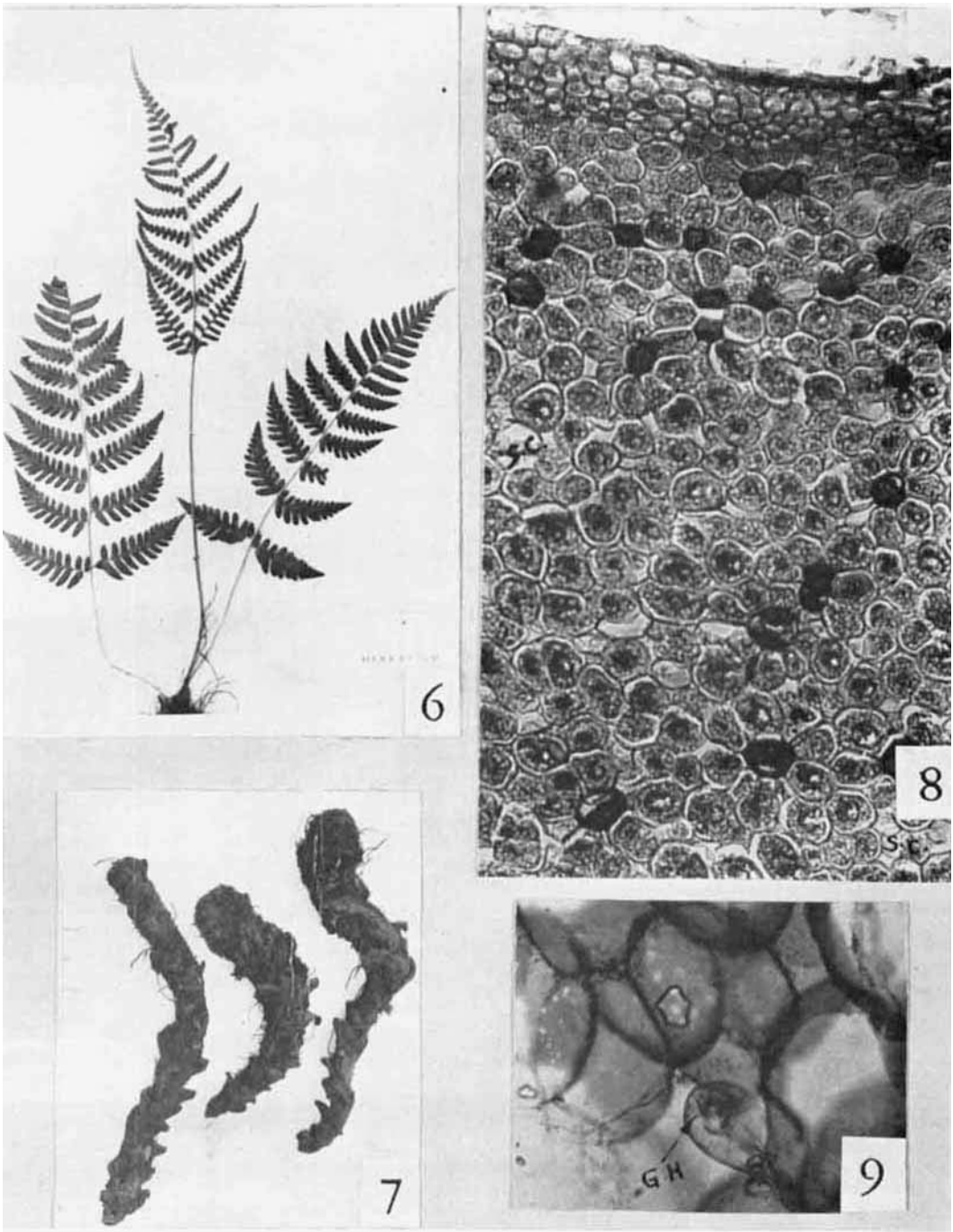


Fig. 3.—*D. cochleata*, plant with two sterile fronds and central fertile one, × 1/5. 7, Rhizome, × 1/2. 8, Sector of T.S. of the rhizome magnified, × 100; S.C., schizogenous cavity. 9, L.S. of rhizome showing a gland in a schizogenous cavity, × 250; G.H., glandular hair.

forming a row on each side of the pinnule near the midvein; indusium is reniform, and persistent.

Macroscopy of the Rhizome.—Rhizomes are flattened-cylindrical, tapering at the base (Fig. 3, part 7), 8–20 cm. long, 1.5–2.5 cm. thick, actual thickness without leaf bases varies from 1–1.5 cm. Externally rufo-castaneous or dull brown, covered by distantly situated frond bases which are 6–11 mm. thick and wrinkled. The entire rhizome and the frond bases are profusely scaly. Fracture is short. Freshly cut surface is pale green, marked by 3–5 cauline meristeles and numerous smaller vascular strands constituting leaf traces. Dark spots of sclerotic nests are abundant in the rhizome and leaf bases. Odor is slight. Taste at first sweetish, later becoming acrid and nauseous.

The ramenta vary in shape from linear-lanceolate to ovate-lanceolate with acuminated apex, each measuring 1–2 cm. × 1–6 mm. Unicellular glands are found all over the surface and the margin. In older scales the marginal cells are somewhat drawn out in groups to form multicellular, long fibrils. In addition, two-celled projections, characteristic of the genus *Dryopteris*, are also present on the margin. The unicellular glands measure 40–50 μ in length, and about 20 μ in diameter at the globular head.

Histology of the Rhizome.—A sector of the transverse section of the rhizome is represented in Fig. 3, part 8. The general histological pattern is the same as described in the earlier species except that numerous dark scattered sclerotic cells or groups of such cells forming sclerotic nests are observed in the ground tissue. In T.S. the number of cortical cells is about 275/mm.². These cells in maceration measure 56–210 × 42–100 μ. Schizogenous cavities are fewer than in the previous species, being only about 25/mm.² as seen in T.S. In longitudinal section, the cavities measure 64–180 × 35–60 μ. Into each cavity project one or two characteristic unicellular glandular hairs (Fig. 3, part 9). The latter are 38–80 μ long including the stalk which is 6–14 μ long with a somewhat swollen base. The diameter of the head measures 24–42 μ. Embedded in the ground tissue are numerous sclerotic cells or selerotic nests, scattered all over but more frequent in the outer cortical region (Fig. 3, part 8), particularly where the frond bases are attached. The sclerotic nests in the outer zone consist of two macrosclereids but those in the pith region are broader and formed of a number of sclereids. The sclereids, which in T.S. appear isodiametric to polygonal in outline with narrow lumen, are elongated cells with pointed or blunt ends and highly pitted walls (Fig. 4*d*). The walls are impregnated with a coloring matter which is responsible for the dark appearance of the nests. Each sclereid measures 250–1275 × 45–120 μ. The number of cauline meristeles varies from 3–5 and each leaf trace is comprised of 10–14 smaller vascular strands.

Histology of the Stipe.—The cortical cells are only slightly broader than those of the rhizome and they number about 200/mm.².

The sclerotic cell/nests are numerous and scattered all over the ground tissue. Each sclerotic nest consists of up to four sclereids. The vascular strands are 10–14, arranged in the form of a closed 'C'. As in the previous species, the two inner bundles are larger than the rest and their xylem possesses anterior hooks.

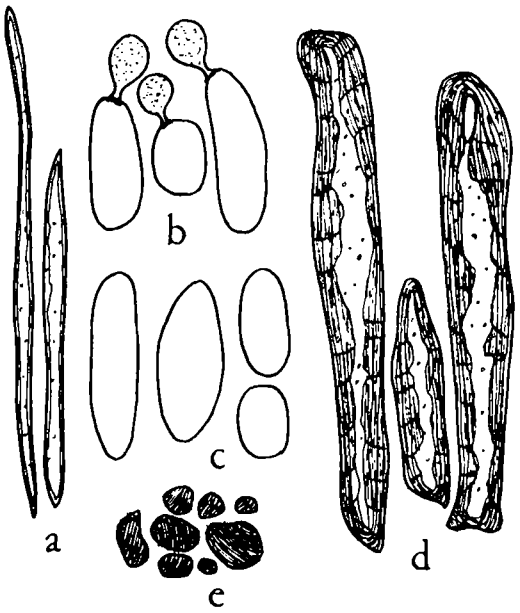


Fig. 4.—*D. cochleata*, tissue macerations. *a*, Hypodermal fibers, × 160; *b*, gland bearing cortical cells, × 160; *c*, cortical cells, × 160; *d*, sclereids × 160; *e*, starch grains, × 700.

SUMMARY

The present investigation provides macroscopic and microscopic descriptions of the rhizome and stipe bases of *Dryopteris barbigera* (Moore) O. Ktze. and *D. cochleata* (Don) C. Chr.

D. cochleata is strongly dimorphic, but *D. barbigera* is monomorphic. The rhizome of the former is flattened-cylindrical with the frond bases distantly situated. That of *D. barbigera* is ovoid, oblong, or elongated in shape with numerous closely arranged frond bases. Rhizomes of both the species are covered all over with ramenta. Size and structure of the ramenta is characteristic in each species.

The histological pattern in both the species is similar except for the presence of scattered dark spots of sclerotic cells and nests in the ground tissue of the rhizome and stipe bases of *D. cochleata* which are absent in *D. barbigera*. Both the species possess schizogenous cavities distributed in the ground tissue, into which project characteristic unicellular secretory glandular hairs. The secretory glands possess longer stalks are *D. barbigera* than in *D. cochleata*. The base of the stalk is slightly swollen only in *D. cochleata*. The number of cauline meristeles and the leaf traces are less in *D. cochleata* than in *D. barbigera*.

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Sensitization of the Pigeon to Epinephrine-Induced Ventricular Fibrillation by a Substituted Propiophenone

By VIIU V. KLEIN, STANLEY E. SRENSEK, and LEHMAN M. LUSKY

The cardiac effects of a substituted propiophenone (U-0882) have been studied in the pigeon. The results obtained and the techniques used indicate that a simple test may be employed to evaluate the cardiac-sensitizing propensities of a compound. The test is based on the biological determination of the quantity of epinephrine required to induce ventricular fibrillation in pigeons which have been pretreated with a usually tolerated parenteral dose of the compound being tested.

PAST INTEREST in cardiac sensitization has been primarily limited to volatile halogenated hydrocarbons in medical anesthesia and industrial toxicology. However, an increasing number of parenterally administered chemical substances have been shown capable of sensitizing the heart to fatal epinephrine-induced ventricular fibrillation (1–4). Moore and Swain (4) recently suggested that compounds under pharmacologic investigation be examined for possible cardiac-sensitizing action in order to prevent the inadvertent induction of ventricular fibrillation. Conventional *in vivo* procedures demonstrating the capability of a substance to sensitize the heart to ventricular fibrillation are unfortunately impractical for routine examination of compounds under investigation because they are expensive, cumbrous, and time-consuming.

Previous studies in this laboratory by Braun and Lusky (5) demonstrated that the action of certain cardiac agents in the pigeon was similar to that in mammals. The pigeon is currently used in the official assay of digitalis (6).

The present communication describes the cardiac effects of alpha-phenoxy-alpha-dimethylaminomethylpropiophenone hydrochloride (hereafter designated as U-0882)[1] in the unanesthetized pigeon. The sensitizing action of this compound on the heart of the dog has previously been reported by Moore and Swain (3).

Although the present paper describes certain toxic manifestations and properties of U-0882, the main purpose of these studies was to devise a feasible method for measuring the cardiac-sensitizing propensities of a compound.

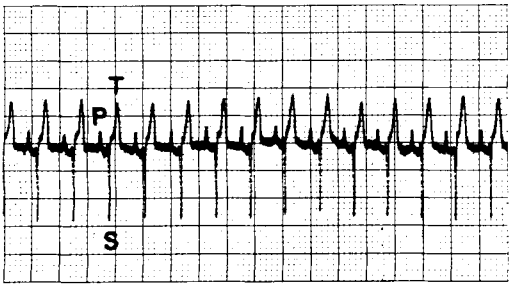


Fig. 1.—Lead II of normal electrocardiogram of pigeon.

EXPERIMENTAL PROCEDURES AND RESULTS

Unanesthetized, common barn pigeons, predominantly of the male sex, ranging in weight from 290 to 400 Gm. were employed in this study. All pigeons were immobilized and the vagi were intact. Artificial respiration was not employed and tracheal cannulation was not performed. Platinum electrodes were inserted into the right wing base, the left wing base, and the left leg of the pigeon. Standard lead II electrocardiograms were taken using a Sanborn Viso-Cardiette, model 51.

Varying doses of U-0882 and epinephrine were administered through an indwelling polyethylene catheter into an alar vein. Each dose was followed immediately by a 0.2-ml. wash of isotonic saline. All doses of epinephrine hydrochloride were so diluted in physiological saline that each of the test dosage levels was administered in a 1 ml./Kg. volume. The U-0882 was administered as a 1.0% solution in physiological saline.

Normal Electrocardiogram, Lead II, of the Pigeon.—The electrocardiogram of a normal, unanesthetized, adult male pigeon taken on lead II is illustrated in Fig. 1. The P, S, and T waves are well defined, but the electrocardiogram differs from that of man in that the pigeon exhibits no Q wave. There is controversy among investigators as to the existence of an R wave in the bird (7, 8).

Induction of Ventricular Fibrillation by U-0882 Alone.—When U-0882 was administered intravenously to groups of 12 pigeons each at dose levels of 20, 30, and 40 mg./Kg., ventricular fibrillation was induced in a few of the unanesthetized birds without the use of epinephrine or other triggering agent. The results show that ventricular fibrillation was induced in 1 of 12 pigeons receiving 20 mg./Kg., in 2 of 12 receiving 30 mg./Kg., and in 4 of 12 pigeons receiving 40 mg./Kg. The 40-mg./Kg. dose caused violent clonic convulsions in 8 of 12 pigeons. The convulsions were fatal to 2

Received June 23, 1961, from the Division of Pharmacology, Bureau of Biological and Physical Sciences, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington 25, D. C.
Accepted for publication July 6, 1961.

[1] The compound U-0882 used in this study was kindly provided by the Upjohn Co., Kalamazoo, Mich.

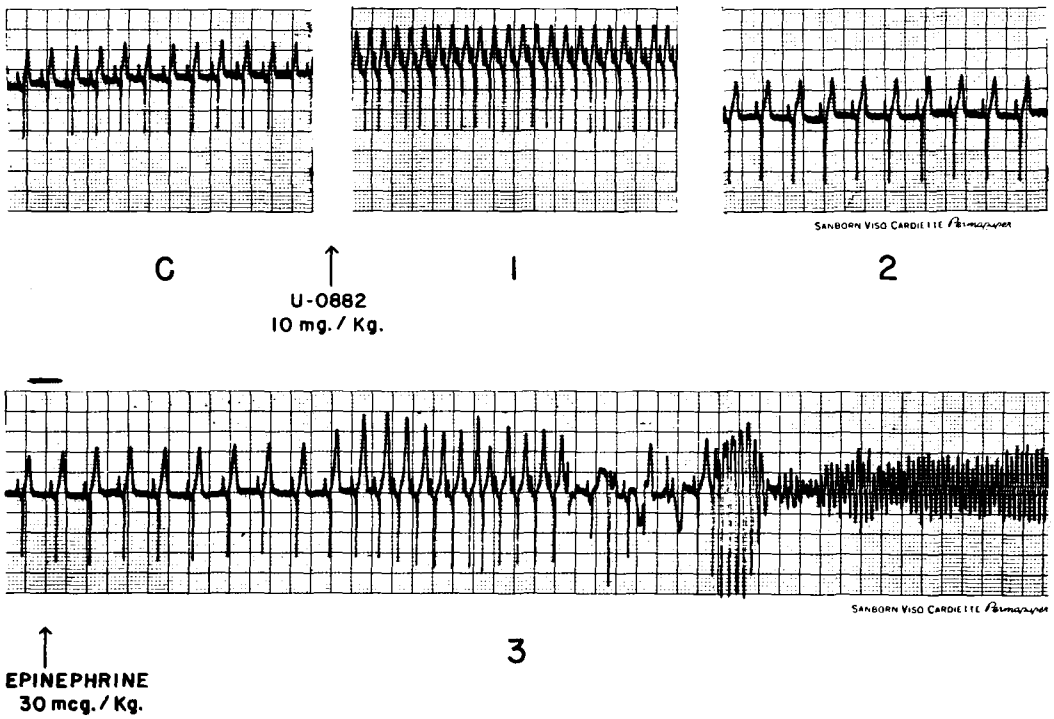


Fig. 2.—Lead II, electrocardiogram of pigeon. C, control; 1, tachycardia following U-0882; 2, bradycardia 2 minutes after U-0882; 3, progression into fibrillation after dose of epinephrine.

of these 8 pigeons; ventricular fibrillation was the cause of death of 2 others, and 4 pigeons recovered from the convulsions. Of the 4 pigeons which did not have convulsions, 2 succumbed from ventricular fibrillation and 2 pigeons did not exhibit any serious toxic effects.

Influence of U-0882 on the Heart Rate of the Pigeon.—The heart rate varied from pigeon to pigeon following the administration of 10 mg. of U-0882/Kg. intravenously. Tachycardia occurred in all of the birds within 1 minute after the injection, but by the end of the second minute, the rate either approximated normal or bradycardia ensued (Fig. 2). The electrocardiograms of 59 pigeons were examined to determine whether the reduction of heart rate following the administration of U-0882 bears a relationship to the incidence of ventricular fibrillation induced by a subsequent injection of epinephrine. The dose of 10 mg. of U-0882/Kg. caused cardiac slowing of varying degree in 43 pigeons and no significant change in rate in 16 pigeons. Following the administration of epinephrine, ventricular fibrillation was induced in 25 of 43 pigeons of the first group and also in 8 of the 16 pigeons of the second group. The apparent higher incidence of fibrillation in the group with the bradycardia was not statistically significant ($p = 0.07$).

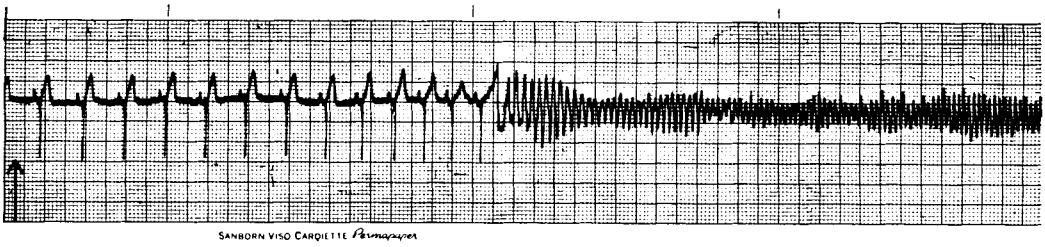
Epinephrine Dose as Index to Cardiac Sensitizing Activity.—Six groups of 12 pigeons each were pretreated with 10 mg. of U-0882/Kg. intravenously. Two minutes later these groups received 3, 5, 10, 20, 30, and 40 mcg./Kg. of epinephrine, respectively. Electrocardiograms were taken to observe any effects on the heart. The electrocardiograms in Fig. 3 illustrate the two observed typical progressions into ventricular fibrillation. In the upper panel the onset of fibrillation is sharp and devoid of ectopic beats. The lower panel, in contrast, shows numerous ectopic beats preceding the onset of ventricular fibrillation in the pigeon. Two of the 72 pigeons on experiment were discarded because fibrillation was induced before the injection of epinephrine by U-0882 alone. Ventricular fibrillation was fatal to all pigeons. No deaths were due to other causes.

Figure 4 shows the dose-response curve obtained in these experiments. The dose of epinephrine (HCl) found to produce ventricular fibrillation in 50% of the pigeons, was calculated by the method of least squares to be 8.3 mcg./Kg. with a range of 6.2–12.8 mcg./Kg. The calculated slope was 1.3 probits per log dose.

In general, the onset of ventricular fibrillation occurred 5 to 10 seconds after the injection of epinephrine. For 15 to 20 seconds after the onset of fibrillation the pigeons lay quietly, respiration appeared normal, and there was no overt indication of the toxic manifestation. This was followed by an arching of the head and neck, brief clonic convulsions, and a final gasp. A similar sequence of events is observed in pigeons after toxic doses of digitalis.

DISCUSSION

The sensitization of the heart to fatal epinephrine-induced ventricular fibrillation by a chemical compound has not been previously described in the pigeon or any other member of the avian genus. The results of the present investigation indicate that the cardiac-sensitizing activity may be evalu-



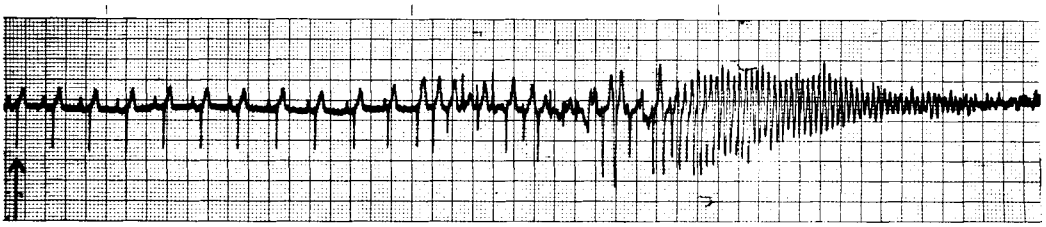


Fig. 3.—Two modes of electrical progression into U-0882–epinephrine ventricular fibrillation.

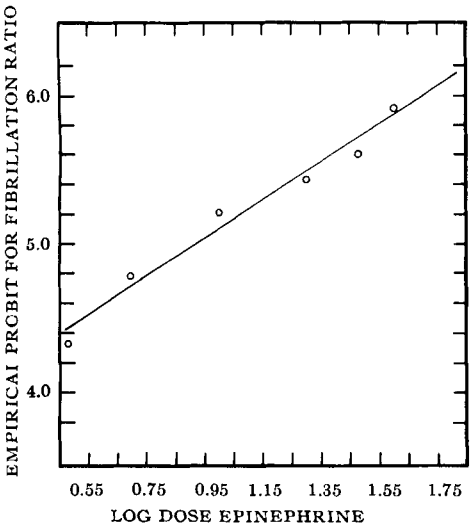


Fig. 4.—Relationship of dosage of epinephrine to incidence of ventricular fibrillation in pigeons sensitized with U-0882.

ated by the dose of epinephrine which will induce fibrillation in 50% of the pigeons previously receiving a usually tolerated dose of the compound under examination.

The observed slope of the dose-response curve obtained from the data of these experiments was 1.3 probits per log dose. This is a relatively flat dose-response curve in comparison with the dose-response curves of various other drugs and their actions. The present data are obviously insufficient to conclude whether this flatness is characteristic of pigeons, epinephrine, U-0882, the combination, or of drug-induced ventricular fibrillation. In any event the low cost of pigeons makes it economically feasible to use relatively large numbers of test animals to attain any desired degree of precision in evaluating the potential of a drug for inducing ventricular fibrillation. The use of the pigeon in cardiac-sensitization studies has other advantages over the use of other species. Artificial respiration often required in other species may not be necessary. The time-saving factor should encourage the use of pigeons. Only 5 to 10 minutes were required for the completion of each individual pigeon test.

SUMMARY

The sensitizing action of a substituted propiophenone, U-0882, on the heart of the pigeon to epinephrine-induced ventricular fibrillation has been demonstrated.

The results of these experiments suggest a simple, rapid, and inexpensive approach to the detection and evaluation of cardiac-sensitizing properties of compounds as a routine toxicity procedure.

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Carbon Dioxide Uptake Studies in Algae Grown in Water and Deuterium Oxide

By MARTIN I. BLAKE†, ARLENE S. KAGANOVE‡, and JOSEPH J. KATZ‡

A procedure is described for studying carbon dioxide uptake in algae using C^{14}-labeled sodium bicarbonate as the source of carbon dioxide. Actively dividing, water grown and deuterium oxide adapted, *Scenedesmus obliquus* and *Chlorella vulgaris* were employed in these studies. Uptake comparisons were made over pH range 6 to 9 using appropriate buffer systems. Uptake was fairly constant in the range pH 6 to 8 for both the aqueous and deuterated algae. Above pH 8 uptake dropped markedly. In general, the deuterated algae showed between 10 and 30% lower uptake than ordinary algae. Greater chlorophyll content is associated with higher carbon dioxide uptake.

RECENT REPORTS (1, 2) indicate that it is possible to culture certain algae in a medium containing better than 99% deuterium oxide. Algae grown under these conditions are a practical source of fully deuterated compounds. A number of monosaccharides and amino acids have been isolated from a hydrolysate of deuterated algae (3). The isolation and characterization of deuterated chlorophylls a and b from chloroplasts of algae and some preliminary experiments on photosynthesis in deuterated algae have also been described (4). The present report deals with further studies on photosynthesis in ordinary and deuterated green algae. Carbon dioxide uptake comparisons are made here between ordinary water and deuterium oxide-grown algae.

The influence of the kinetic isotope effect on photosynthesis is probably among the most significant differences in the culturing of algae in water and deuterium oxide. It is hoped that such comparative studies as are reported here will aid in a better understanding of the complexities involved in the conversion of carbon dioxide to organic compounds by the photosynthetic process.

EXPERIMENTAL

Algae Cultures.—*Scenedesmus obliquus* and *Chlorella vulgaris* were grown autotrophically as stock cultures in 250-ml. culture flasks; there were two such cultures for each organism in both aqueous and deuterated media. The composition of the media has previously been given (2). The volume of the cultures was maintained at 125 ml. The flasks were kept on a mechanical shaker and the algae were continuously illuminated by overhead fluorescent lighting at an intensity of about 700-ft. candles measured at the level of the algae culture. Carbon dioxide was fed as a 5% mixture in nitrogen. The cultures were maintained at room temperature (25–27°).

An algae suspension for an experiment was prepared by centrifuging the harvested cells for 5 minutes at full speed in an International clinical centrifuge. The supernatant liquid was discarded, and the cells were washed once with water or deuterium oxide. The cells were resuspended in fresh solvent, and the packed cell volume was determined by centrifuging for 20 minutes. In all experiments a 1% suspension of algae in buffer solution was prepared by suspending 0.5 ml. of packed cells in 50 ml. of buffer. Only algae from rapidly growing cultures were used in these studies since older cultures were far less active in photosynthesis.

Photosynthesis Flask.—A specially constructed flask was used for the carbon dioxide uptake studies. A ground-glass standard-taper 125-ml. Erlenmeyer flask (male joint) was fitted with a cap (female joint) having a tapered opening extending about 2 cm., over which was fitted a rubber injection-type stopper. Aliquots of the solution within the flask were conveniently removed by hypodermic syringe without loss of carbon dioxide from the system. Experimentation showed that no measurable diffusion of carbon dioxide through the rubber stopper occurred over an 8-hour period. The cap was held tightly to the flask by springs fixed to glass prongs. A circular glass shelf, about 1 cm. in diameter and having a lip 4 mm. high, was fitted to the inner wall of the flask just above the 50-ml. mark. The flask was fitted with a jacket, covering the base and side of the flask above the 50-ml. mark. The algae suspension was maintained at 26° by passing water from a constant temperature water bath through the jacket. The temperature of the algae suspension was determined by inserting a thermometer into the well provided on the side of the flask. Figure 1 shows a diagram of the flask.

$NaHC^{14}O_3$ Stock Solution.—A stock solution of C^{14}-labeled sodium bicarbonate was prepared by dissolving 2 mc. of isotope in 4 ml. of pH 9 phosphate buffer solution. The solution was assayed by diluting a 25-λ aliquot to the mark of a 50-ml. volumetric flask. A 100-λ aliquot of this solution was added to 15 ml. of liquid scintillator in a 5-dr. vial and the system was counted in a Tri-Carb

Received April 28, 1961, from the Chemistry Division, Argonne National Laboratory, Argonne, Ill.
Accepted for publication July 27, 1961.
Based on work performed under auspices of the U. S. Atomic Energy Commission.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
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‡ Chemistry Division, Argonne National Laboratory, Argonne, Ill.
The authors express their gratitude to Dr. Martin Kilpatrick for his helpful suggestions and to Mrs. Mary Thomas for her technical assistance in certain phases of this study.

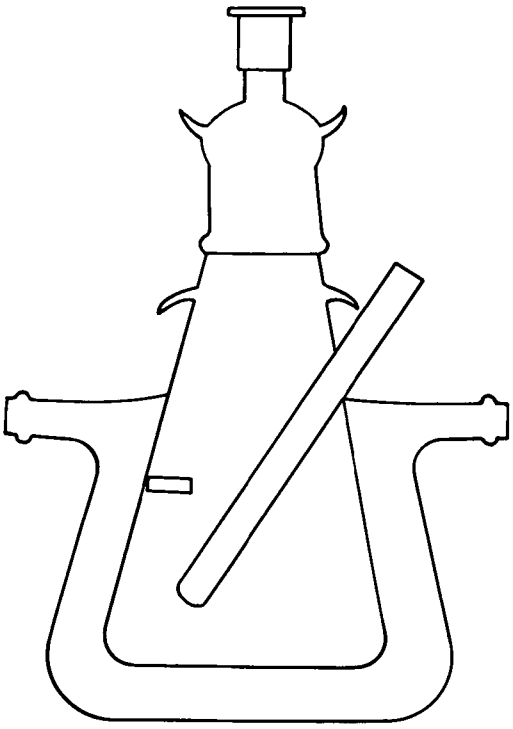


Fig. 1.—Schematic diagram of photosynthesis flask.

liquid scintillation spectrometer (model 314). A count rate of about 30,000 counts/min./100 λ was observed. This was compared to a standard.

General Procedure.—In all photosynthetic experiments 50 mg. of sodium bicarbonate and 25 λ of $NaHC^{14}O_3$ solution (12.5 μc.) were placed on the shelf of the photosynthesis flask. The algae suspension, prepared as described above, was added to the flask, below the shelf, with the aid of a long stem funnel. The flask was capped and, at zero time, was shaken vigorously to distribute the sodium bicarbonate and isotope throughout the algae suspension. The flask was placed on the shaker where the algae were permitted to photosynthesize. At 90-minute intervals for a total of 6 hours a small aliquot was removed from the flask by hypodermic syringe, centrifuged for 3 minutes, and 100 λ of the supernatant solution was counted.

Calibration Curves.—*Aqueous System.*—At pH 7.0 and below, a substantial portion of the sodium bicarbonate is present as carbon dioxide, part of which is in solution and part in the gas phase. The equilibria involved may be illustrated as follows

$[CO_2]$ gas phase

$\Downarrow\Uparrow$

$[CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-]$ aqueous phase

$\Downarrow\Uparrow$

[CO_2 taken up by algae[1]]

A calibration curve was necessary in order to determine the amount of carbon dioxide in the gas phase and the carbon dioxide plus other species in the liquid phase from a measurement of the C^{14} content of an aliquot of the liquid phase. Theoretically, this information could be obtained by calculation, but some of the necessary constants are not available for the deuterium oxide system. An empirical calibration curve supplied a more certain answer.

To the shelf of each of a series of 5 flasks was added respectively: 50 mg., 40 mg., 30 mg., 20 mg., and 10 mg. of sodium bicarbonate. This was followed by the addition of 25 λ of $NaHC^{14}O_3$ solution to the shelf of each flask. Fifty milliliters of buffer solution was added to the flasks below the shelf. The flasks were capped and shaken mechanically for 90 minutes. An aliquot of 100 λ was counted. Since the specific activity (counts/min./mg.) of the sodium bicarbonate was known, the amount of sodium bicarbonate in the aqueous phase was readily determined. The amount of carbon dioxide, as sodium bicarbonate equivalent, in the gas phase was then known. As an illustration, when 50 mg. of sodium bicarbonate and 25 λ of $NaHC^{14}O_3$ solution were used in pH 6.0 buffer, the specific activity was 300,000 counts/min./mg. of sodium bicarbonate. After equilibration, the activity in the liquid phase was observed to be 8,500 counts/min./100 λ or 4,250,000 counts/min./50 ml. This represented 14.2 mg. of sodium bicarbonate in the aqueous phase and 35.8 mg. of sodium bicarbonate equivalent in the gas phase as carbon dioxide. Corresponding values were obtained for the other weights of sodium bicarbonate which were employed. A calibration curve was constructed by plotting mg. of sodium bicarbonate equivalent in the gas phase *vs.* mg. of sodium bicarbonate in the aqueous phase. A straight line was obtained which passed through the origin.

For pH 6.0 a phosphate buffer system (KH_2PO_4 + NaOH) was used. For pH 7.0 a tris(hydroxymethyl)aminomethane-hydrochloric acid buffer system was used. The calibration curve for pH 7.0 with this buffer was not linear. More carbon dioxide was bound in the liquid phase than expected, due probably to the formation of carbamate. Since the calibration curves apply only to the vessels and conditions used in this study, the curves are not given here.

The "tris" buffer system was used for those studies at pH 8.0, 8.5, and 9.0. Since the carbon dioxide in the gas phase at pH values of 8.0 and above is negligible, a calibration curve was not necessary here. The observed activity in the aqueous phase of blank runs did not deviate from the theoretical value, and the direct count of the liquid phase was all that was required at these pH values.

Deuterium Oxide System.—The equilibria involved in the carbon dioxide uptake studies with deuterium oxide-grown algae are essentially the same as those involved in the aqueous system except that deuterium replaces hydrogen. Calibration curves were prepared as described for the aqueous system. The buffer solution corresponding to pH 6.0 was prepared from NaOD and KD_2PO_4. The apparent pH as observed with a Beckman pH-meter (model G) was 5.9. The buffer solutions in deuterium oxide (D_2O) corresponding to pH values of 7.0, 8.0, 8.5, and 9.0 were prepared from tris(hydroxymethyl)-aminomethane (in which all of the exchangeable hydrogen was replaced by deuterium) and DCl. All buffers in D_2O were prepared using the same stoichiometry as for the H_2O buffers. The apparent pH was measured with the pH-meter and an ap-

[1] Species other than carbon dioxide may be involved.

propriate correction (5) to the reading was made. The pD values of the buffer solutions in D_2O corresponding to the aqueous buffer values were 6.3, 7.3, 8.3, 8.8, and 9.3.

Effect of pH on Carbon Dioxide Uptake.—Carbon dioxide (or bicarbonate) uptake was studied over a 6-hour period. The algae were agitated continuously at 26° at a light intensity of 700-ft. candles. Comparison was made between algae grown in water and deuterium oxide over a pH range of 6.0 to 9.0. At pH values (and corresponding pD values) of 8.0, 8.5, and 9.0% uptake was determined directly from the counts/min./100-λ aliquot of the aqueous phase at zero time and the counts/min./100-λ aliquot after an interval of photosynthesis. It was assumed that at these pH values there was a negligible amount of carbon dioxide in the gas phase. For pH (and pD) values of 6.0 and 7.0 the carbon dioxide in the gas phase must be taken into account. The total count rate in the liquid phase was determined from the count rate in a 100-λ aliquot of the solution. The amount of bicarbonate in the liquid phase was readily determined, and the amount of sodium bicarbonate equivalent in the gas phase was ascertained from the calibration curve. The sum in the aqueous phase and the gaseous phase was then subtracted from the original amount. This represented the amount taken up by the algae. Per cent uptake was then calculated. The data for these experiments are shown in Figs. 2–5.

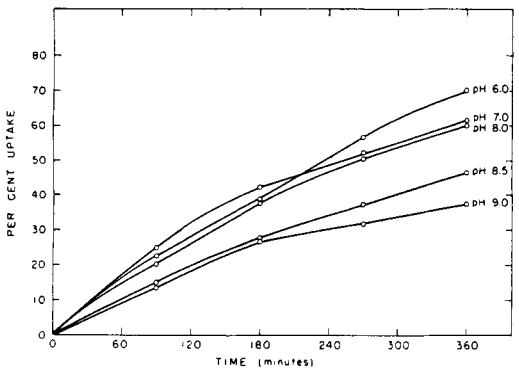


Fig. 2.—Uptake of $NaHC^{14}O_3$ by ordinary *C. vulgaris* in water as a function of pH and time.

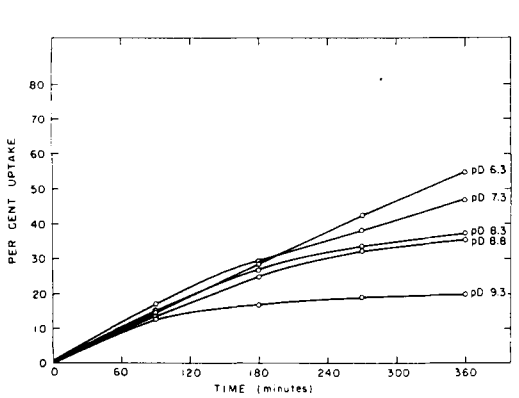


Fig. 3.—Uptake of $NaHC^{14}O_3$ by deuterated *C. vulgaris* in deuterium oxide as a function of pD and time.

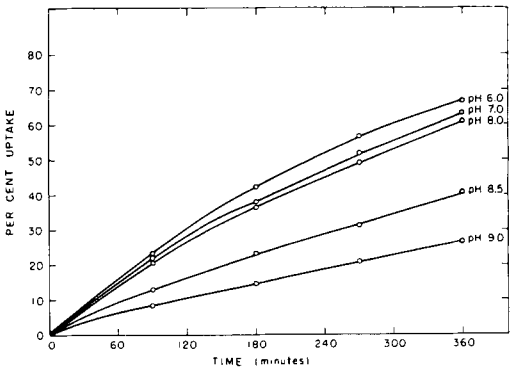


Fig. 4.—Uptake of $NaHC^{14}O_3$ by ordinary *S. obliquus* in water as a function of pH and time.

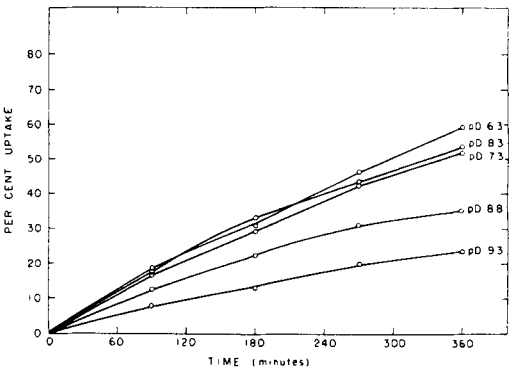


Fig. 5.—Uptake of $NaHC^{14}O_3$ by deuterated *S. obliquus* in deuterium oxide as a function of pD and time.

Radioactive Excretion Products.—Tolbert and Zill (6) have reported that at pH values greater than 4.5 glycolic acid is an excretion product of *Chlorella*. Experiments were therefore conducted to determine whether significant amounts of glycolic acid or other radioactive organic compounds were excreted into the medium in these studies. Samples of algae suspensions were centrifuged and the supernatant liquid transferred to a second tube. Concentrated hydrochloric acid was added (50 λ) and the solution was flushed with nitrogen. A 250-λ aliquot was then counted. In no instance was the count rate sufficiently above background to indicate the presence of significant amounts of radioactive metabolic products. This was further confirmed by applying the color reaction with 2,7-dihydroxy naphthalene as described by Calkin (7) and used by Tolbert and Zill in their work.

Chlorophyll Content.—The chlorophyll content of the algae used in this study was determined. Exactly 0.5 ml. of wet packed cells (representing 0.1 Gm. of dried cells) was transferred to a 15-ml. centrifuge tube. About 10 mg. of sodium bicarbonate was added, followed by the addition of 10 ml. of actively boiling water. The tube was then placed into boiling water for 1 minute, cooled under tap water, and centrifuged. The supernatant liquid was discarded. Ten milliliters of a mixture of ethyl ether (2 volumes) and methanol (1 volume) was added, and the tube was shaken vigorously until its contents were

thoroughly mixed. The tube was centrifuged and the supernatant liquid was transferred to a separator. This extraction procedure was repeated twice. An additional 30 ml. of ether-methanol solvent mixture was added to the separator and the solution was extracted several times with saturated salt solution, followed by extraction with distilled water. The chlorophyll layer was transferred to a 100-ml. volumetric flask and was diluted to the mark with ether. Absorption was determined with a Cary model 14 recording spectrophotometer at 660.0 and 642.5 mμ, and both total chlorophyll and chlorophyll a were calculated by the method of Comar (8). The data are recorded in Table I.

TABLE I.—DETERMINATION OF TOTAL CHLOROPHYLL AND CHLOROPHYLL a IN DEUTERIUM OXIDE AND WATER-GROWN ALGAE

Algae	Total Chlorophyll,[a] %	Chlorophyll a,[a] %	Total Chlorophyll D_2O/H_2O	Chlorophyll a, D_2O/H_2O
C. vulgaris				
H_2O	3.44	2.57	0.779	0.747
D_2O	2.68	1.92	...	...
S. obliquus				
H_2O	2.45	1.84	0.955	0.880
D_2O	2.34	1.62	...	...

[a] Grams per 100 Gm. of dried algae.

DISCUSSION

The availability of fully deuterated algae has made possible a comparative study of photosynthesis in both deuterium oxide-adapted and water-grown algae. The replacement of hydrogen by deuterium probably affects the relative rates of reactions involved in photosynthesis, and does not interfere with the pathways by which carbon dioxide is converted to organic compounds. This is substantiated by the fact that the same metabolites, but which contain deuterium where hydrogen is normally found, have been isolated (3). Identical, but deuterated, chloroplast pigments have also been extracted (4). In other studies (9) essentially similar radioautograms have been obtained using the techniques described by Bassham and Calvin (10). Although similar compounds were identified, it is quite possible that the relative abundances may differ. The course of photosynthesis, at least in its earlier phases, appears to be substantially the same in both varieties of algae.

Figures 2–5 show carbon dioxide uptake by deuterated and water-grown algae as a function of time and pH (or pD). It is apparent that the water-grown algae take up carbon dioxide to a greater extent than the deuterated algae over a 6-hour study period. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms. Although there is a greater disparity in uptake between the aqueous and deuterated *Chlorella*, it is interesting to note that in an earlier report (2) it was shown that adaptation in deuterium oxide occurs more readily with *Chlorella* than with *Scenedesmus*.

In general, under the conditions of the experiments described here, deuterated algae showed between 10 and 30% lower uptake than ordinary algae. The influence of variation in pH on bicarbonate uptake by ordinary and deuterated algae was studied. Since the electrochemistry of D_2O and H_2O is different, it is essential to distinguish between kinetic isotope effects and changes in uptake rate due to pH sensitivity. Solutions in H_2O and D_2O made up stoichiometrically identical will have different ion concentrations and activities. Suitable corrections were made for the observed values in the deuterium oxide systems. Uptake was fairly constant in the range pH 6 to 8 for both ordinary and deuterated algae. The uptake is thus insensitive to pH changes in this range. Therefore, differences in uptake are attributable to metabolic effects involving deuterium rather than pH sensitivity. Above pH 8, uptake dropped markedly and at pH 9, the algae showed signs of damage in a short period of time.

The question of whether carbon dioxide, bicarbonate ion, or both are utilized by algae as a source of carbon has been considered by numerous workers. Evidence in the literature is very conflicting. This matter is discussed by Krauss (11). Emerson and Green (12) claim that sufficient carbon dioxide is released in carbonate-bicarbonate buffer systems for photosynthesis even at high pH values. The equilibrium curve shows that at pH values above 8, only traces of carbon dioxide are present and, therefore, it appears improbable that sufficient carbon dioxide is released to satisfy the requirements of algae cultures. Osterlind (13) has found that *Scenedesmus quadricauda* assimilates bicarbonate ion while *Chlorella pyrenoidosa* does not. On the basis of the present report it seems probable that bicarbonate is acceptable to the algae in the alkaline pH region. For example, at pH 8.0, where free carbon dioxide is practically negligible, satisfactory photosynthesis was observed. At pH 8.5 and above, the alkalinity of the solution was found to have a detrimental effect on photosynthesis and growth. One can also speculate that since a "tris" buffer was employed in much of this work, carbamate may also be capable of being absorbed by the algae. Finally, the possibility exists that algae may possess two independent mechanisms for assimilating carbon: (*a*) one for carbon dioxide and (*b*) another independent path for bicarbonate ion. In an occasional run it was noted that while the algae consumed carbon dioxide normally at pH values around 7, they were refractory to bicarbonate uptake at pH 8.

Preliminary experimentation indicated that the absolute amount of sodium bicarbonate consumed by the algae over a time period was independent of the initial concentration. This was studied over the range of starting concentrations from 25 to 200 mg.

Experiments were conducted to determine whether significant amounts of glycolic acid, as reported by Tolbert and Zill (6), or other C^{14}-labeled compounds were excreted into the medium under the conditions of these studies. Tolbert and Zill used essentially weightless amounts of sodium bicarbonate while in the present study macroscopic quantities of bicarbonate, mainly nonlabeled, were employed. This precluded carbon dioxide starvation which may be responsible for glycolic acid excretion. Radioactive excretion products were not detected in our studies.

The relationship of chlorophyll content and carbon dioxide uptake was examined. Total chlorophyll and chlorophyll a were determined for both the aqueous and deuterated algae. As shown in Table I, the ratio of chlorophyll content for the deuterated to that of the aqueous algae approximates the carbon dioxide uptake relationships shown by the uptake curves. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms, which is consistent with the results from the uptake studies.

The procedure described in this report for studying carbon dioxide uptake is a simple one and reproducible results within 5% were obtained when duplicate runs were conducted. Further work is currently in progress in this laboratory to extend these studies to include the effect of variation in light intensity and temperature on the photosynthesis of deuterated algae.

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Determination of Barbiturates in Urine Containing Salicylate

By DONALD M. STOKES, WALTER J. R. CAMP, and ERNST R. KIRCH

A procedure is described in which urine pigments, salicylate, and other chromogenic materials which preclude spectrophotometric evaluation of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from a chloroform solution by selective adsorption.

THE EXTRACTION from urine specimens of salicylate and other substances together with barbiturates has been a problem frequently encountered during toxicologic analysis. At the present time, the quantitative determination of barbiturates in biological material is best accomplished by means of ultraviolet spectrophotometry (1, 2). Analytical procedures commonly employed involve preliminary extraction of the malonylurea derivative from the specimen. by an immiscible solvent such as ether or chloroform. The spectral characteristics of the malonylurea molecule are most definitive in alkaline solution, which may be obtained by re-extraction of the barbiturate from an immiscible solvent into aqueous alkali. Normal physiological constituents which absorb strongly in the ultraviolet region and other drugs including salicylates are often extracted along with the barbiturate. The presence of these nonbarbiturate chromogens in the final alkaline extract precludes definitive spectrophotometric evaluation of the malonylurea compound unless steps are taken to remove the interference.

The attempts by various investigators to eliminate interfering substances by use of lengthy, complicated extraction procedures which resulted in loss of sensitivity, low recoveries, and failure to eliminate nonbarbiturate chromogens have been criticized (3). Differential spectrophotometric techniques (3–5) in which determination of barbiturate is based on the differences in the ultraviolet absorption spectra of strongly alkaline and pH 10–10.5 buffered solutions represent a significant improvement over older methods employing conventional spectrophotometry. It has been reported (3) that ultraviolet-absorbing substances including the drugs

Received April 28, 1961, from the University of Illinois, College of Pharmacy, Chicago 12.
Accepted for publication July 8, 1961.
Abstract of thesis submitted to the Graduate College by D. M. Stokes in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Chemistry, Chicago Professional Colleges, University of Illinois.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.

The relationship of chlorophyll content and carbon dioxide uptake was examined. Total chlorophyll and chlorophyll a were determined for both the aqueous and deuterated algae. As shown in Table I, the ratio of chlorophyll content for the deuterated to that of the aqueous algae approximates the carbon dioxide uptake relationships shown by the uptake curves. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms, which is consistent with the results from the uptake studies.

The procedure described in this report for studying carbon dioxide uptake is a simple one and reproducible results within 5% were obtained when duplicate runs were conducted. Further work is currently in progress in this laboratory to extend these studies to include the effect of variation in light intensity and temperature on the photosynthesis of deuterated algae.

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Determination of Barbiturates in Urine Containing Salicylate

By DONALD M. STOKES, WALTER J. R. CAMP, and ERNST R. KIRCH

A procedure is described in which urine pigments, salicylate, and other chromogenic materials which preclude spectrophotometric evaluation of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from a chloroform solution by selective adsorption.

THE EXTRACTION from urine specimens of salicylate and other substances together with barbiturates has been a problem frequently encountered during toxicologic analysis. At the present time, the quantitative determination of barbiturates in biological material is best accomplished by means of ultraviolet spectrophotometry (1, 2). Analytical procedures commonly employed involve preliminary extraction of the malonylurea derivative from the specimen. by an immiscible solvent such as ether or chloroform. The spectral characteristics of the malonylurea molecule are most definitive in alkaline solution, which may be obtained by re-extraction of the barbiturate from an immiscible solvent into aqueous alkali. Normal physiological constituents which absorb strongly in the ultraviolet region and other drugs including salicylates are often extracted along with the barbiturate. The presence of these nonbarbiturate chromogens in the final alkaline extract precludes definitive spectrophotometric evaluation of the malonylurea compound unless steps are taken to remove the interference.

The attempts by various investigators to eliminate interfering substances by use of lengthy, complicated extraction procedures which resulted in loss of sensitivity, low recoveries, and failure to eliminate nonbarbiturate chromogens have been criticized (3). Differential spectrophotometric techniques (3–5) in which determination of barbiturate is based on the differences in the ultraviolet absorption spectra of strongly alkaline and pH 10–10.5 buffered solutions represent a significant improvement over older methods employing conventional spectrophotometry. It has been reported (3) that ultraviolet-absorbing substances including the drugs

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dicoumarol, diphenylhydantoin, and sulfadiazine, which appear in the final alkaline extract, do not interfere with barbiturate determination by differential spectrophotometry because they have the same absorption in strong alkali and in pH 10.5 solution. But salicylate, like barbiturate, exhibits spectral differences (6) dependent on pH and must be removed from a sample before quantitative analysis of the malonylurea derivative can be made.

Although it has been claimed (3, 4, 6) that separation of barbiturate from salicylate can be accomplished by extraction from phosphate-buffered aqueous solution at pH 7.4, the present authors have been unable to secure complete separation of the two compounds by this method. Furthermore, extraction with ether or chloroform of many urine specimens at various pH levels ranging from less than 1 to 7.4 has been found to result in subsequent alkaline extracts which were often highly colored and contained such large amounts of nonspecific chromogenic material that differential spectrophotometric evaluation was not possible.

In this investigation, a reasonably short, simple procedure was employed in which salicylate as well as urinary pigments and chromogens were removed from a chloroform solution by means of a selective adsorption column and barbiturates were eluted quantitatively from the column in sufficient purity to permit determination by automatic differential ultraviolet spectrophotometry.

Spectrophotometric Determination of Barbiturates.—The ultraviolet absorption spectrum of 5,5-disubstituted malonylurea derivatives in strong alkali such as 0.45 *N* sodium hydroxide exhibits a maximum at 252–255 mμ and a minimum at 234–237 mμ. In a buffer solution at pH 10–10.5 there occurs a higher maximum at 238–240 mμ with no minimum (3, 4). The difference between the absorbance in strong alkali and that in the buffered solution is greatest at 260 mμ, decreasing through zero at 247–250 mμ to a maximum negative at 235–240 mμ, and increasing through zero at 227–230 mμ to a positive difference at lower wavelengths. The absorbance difference at 260 mμ has been employed to measure the concentration of barbiturate solutions by differential spectrophotometry (3, 4).

Recently, the differential spectrophotometric technique has been adapted to utilize the automatic ratio-recording features of the Beckman model DK-2 spectrophotometer by Williams and coworkers, who have published procedures for determination of both barbiturates (5) and salicylates (6). The method is based on the fact that in alkaline solution salicylate, like barbiturate, can exist in two different ionic states which give rise to two different ultraviolet spectra, depending on the pH of the solution. The instrument automatically substracts one spectrum from the other and draws a composite curve.

The composite curves shown in Fig. 1 are representative of the differential absorption spectra of 5,5-disubstituted malonylurea derivatives and were obtained using various standard concentrations of secobarbital. In each case the 0.45 *N* sodium hydroxide solution was scanned against the pH 10–10.5 solution as reference, using the differential absorbance range (−0.3 to +0.7) of the Beckman model DK-2 spectrophotometer as described by Williams and Zak (5). The maximum and minimum absorption peaks, occurring at 260 and 238 mμ, respectively, represent the corresponding absorbance differences between strongly alkaline and pH 10–10.5 spectra.

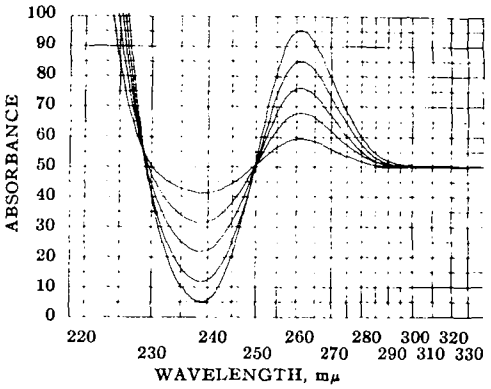


Fig. 1.—Differential absorption spectra of standard solutions of secobarbital in concentrations of 4.994, 9.988, 14.98, 19.98, and 24.97 mcg. per ml.

Salicylate in 0.45 *N* sodium hydroxide, when read against 0.45 *N* sodium hydroxide as a reference blank, exhibits a maximum at 300 and a minimum at 267 mμ. In a buffered solution at pH 10–10.5 there occurs a maximum at 295 and a minimum at 257 mμ. This spectral shift with change in pH gives rise to the differential spectrum. The composite spectrum of salicylate exhibits maxima at 318 and 246 mμ and minima at 283 and 233 mμ (6).

When both barbiturate and salicylate are present in the solutions scanned in the spectrophotometer the differential spectrum exhibits the 318 mμ peak characteristic of salicylate, but the shorter wavelength peaks for barbiturate and salicylate are replaced by a single absorption maximum occurring at about 253 mμ (6). The effect of the presence of salicylate on the differential absorption spectrum of barbiturate is demonstrated in Fig. 2. The composite curve illustrated here was obtained using a solution of salicylate and pentobarbital, each being present in a concentration of 20 mcg. per ml. The composite barbiturate-salicylate spectrum is inadequate for definitive determination of the malonylurea derivative, although quite accurate estimation of the salicylate concentration is possible using the 318 mμ peak (6). Thus it is imperative that salicylate be removed before definitive determination of the barbiturate content of a sample can be made.

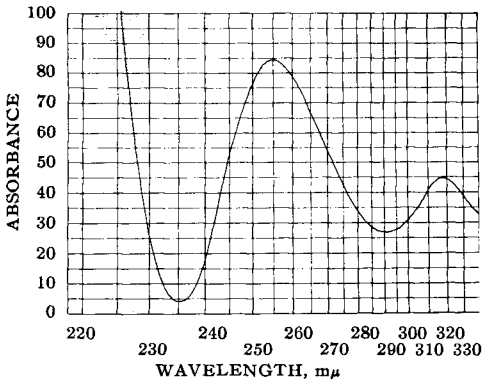


Fig. 2.—Differential absorption spectrum obtained from a solution containing both salicylate and pentobarbital in concentrations of 20 mcg. per ml.

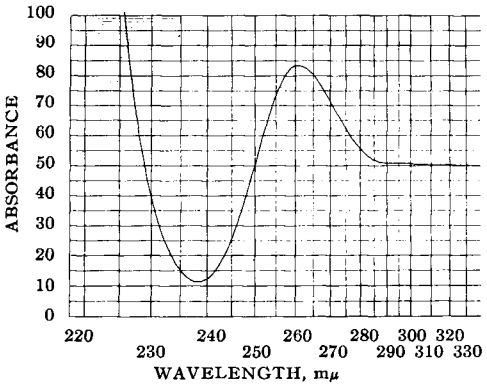


Fig. 3.—Differential absorption spectrum of secobarbital recovered from a Florisil column. A mixture of 10.0 ml. of standard secobarbital solution, 19.98 mcg. per ml., and 10 ml. of urine containing 20 mg. of salicylate was extracted.

A typical differential spectrum obtained on barbiturate recovered from a Florisil[1] adsorption column by the procedure described in this investigation is illustrated in Fig. 3. An extract of a mixture of 10.0 ml. of standard secobarbital solution, 19.98 mcg. per ml., and 10 ml. of urine containing 20 mg. of salicylic acid was purified by passage through the column followed by elution of the barbiturate. The quantitative recovery of secobarbital as well as the effectiveness of the method in removing salicylate and urinary chromogenic material is indicated by comparison of Fig. 3 with the curve obtained on the corresponding standard secobarbital solution shown in Fig. 1.

EXPERIMENTAL

Reagents.—Florisil, 60/100 mesh; diethyl ether, anhydrous reagent; chloroform, reagent grade, redistilled; methanol, anhydrous reagent; hydrochloric acid (A.R.) solution, approximately 6 *N*; sodium hydroxide (A.R.) solution, 0.45 *N*; ammonium chloride (A.R.) solution, 16%.

[1] Florisil is a granular magnesia-silica gel adsorbent supplied by the Floridin Co., Tallahassee, Fla.

Standardization of Barbiturate Solutions.—Standard solutions of the representative 5,5-disubstituted malonylurea derivatives amobarbital, pentobarbital, phenobarbital were prepared as follows: a pure sample of each barbiturate was dried for at least 1 week under vacuum over phosphorus pentoxide. A portion close to 0.25 Gm. was weighed accurately and dissolved in 0.45 *N* sodium hydroxide to make 500.0 ml. of stock solution. Standard solutions ranging in barbiturate concentration from 5 to 30 mcg. per ml. in 0.45 *N* sodium hydroxide were immediately prepared from the stock solution and subjected to differential spectrophotometry. To 3.0 ml. of the standard solution, 0.5 ml. of 0.45 *N* sodium hydroxide was added. A second 3.0-ml. portion of the standard was mixed with 0.5 ml. of 16% ammonium chloride to obtain a buffered solution of pH 10–10.5. The strongly alkaline solution was scanned as sample against the buffered solution as reference, using the differential absorbance range of the Beckman model DK-2 spectrophotometer as described by Williams and Zak (5).

The differential absorbance spectra of various standard concentrations of secobarbital are shown in Fig. 1. Similar curves were obtained on standard solutions of amobarbital and pentobarbital. Although the value of the differential absorbance maximum at 260 mμ was found to be nearly proportional to the concentration, much better linearity resulted when the differences in absorbance between the maximum at 260 mμ and the minimum at about 238 mμ were plotted as a function of the concentration. Calibration curves (Fig. 4) in which the absorbance differences ($A_{max} - A_{min}$) are graphed against concentration demonstrate adherence to the Beer-Lambert law found for each of the barbiturates tested.

Preparation of Adsorption Column.—A small piece of glass wool was fitted into the bottom of a glass tube having the approximate dimensions 65 cm. in height and 1 cm. in inside diameter. Five grams of Florisil was packed fairly loosely to a height of about 10 cm. in the tube by gentle tapping. Another small piece of glass wool was placed on top

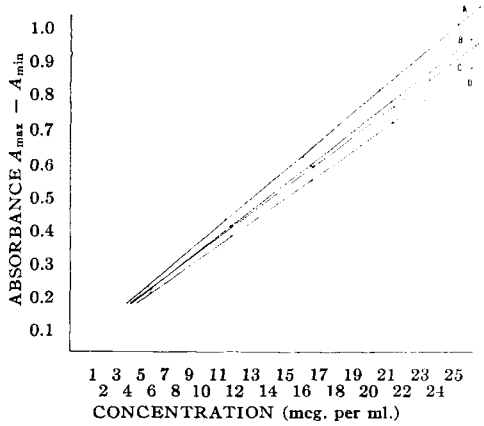


Fig. 4.—Absorbance *vs.* concentration curves for standard solutions of four common barbiturates. *A*—Amobarbital, *B*—phenobarbital, *C*—pentobarbital, *D*—secobarbital.

TABLE I.—RECOVERY OF PENTOBARBITAL FROM PURE SOLUTIONS BY USE OF FLORISIL COLUMNS

Concn., mcg. per ml.	Absorbance: Standard A_{max}	Standard A_{min}	Standard $A_{max} - A_{min}$	Eluate A_{max}	Eluate A_{min}	Eluate $A_{max} - A_{min}$	Recovery, %
5.024	0.609	0.412	0.197	0.609	0.415	0.194	98.5
10.05	0.700	0.305	0.395	0.701	0.310	0.391	99.0
20.10	0.891	0.115	0.776	0.887	0.110	0.777	100.1

of the adsorbent and lightly pressed into place with a glass rod. The column so prepared was ready for use.

Isolation of Barbiturate from Urine.—A 10.0-ml. sample of urine was acidified to pH 1 or below by addition of 3–4 ml. of 6 *N* hydrochloric acid and extracted with ether in three successive portions of about 40 ml. each. The ether phases were carefully separated, combined, and evaporated to dryness on a 70° water bath with the aid of a gentle stream of air. Approximately 10 ml. of redistilled chloroform was added to the dry residue thus obtained and the mixture was warmed on the water bath to facilitate solution of the barbiturate. The Florisil column was treated with 20 ml. of chloroform to initiate solvent flow and wash out any loose particles of adsorbent. The chloroform extract was then placed on the column and followed by two additional 10-ml. portions of chloroform similarly employed to insure complete transfer of barbiturate to the adsorption column. At no time was the liquid level in the column allowed to fall below the upper surface of the adsorbent.

As the developing solvent flowed through the column, a yellow color band was formed within the uppermost part of the adsorbent and a clear, colorless effluent was obtained. The rate of flow varied between 0.5 and 3 ml. per minute, depending on the height of the liquid in the column. When the level of the chloroform was 1 or 2 cm. above the surface of the Florisil, 25 ml. of 10% (by volume) methanol in chloroform was carefully added to the column to elute the barbiturate. The resulting colorless eluate was collected in the receiver containing the chloroform effluent. Little if any diffusion of the urine pigment in the colored band was discernible during elution and salicylate, if present, was retained on the adsorption column.

After the eluant had ceased to flow, the methanol-chloroform solution was evaporated to dryness on a 70° water bath with the aid of a gentle stream of air. The resulting dry residue was subsequently taken up in 10.0 ml. of 0.45 *N* sodium hydroxide and stirred to dissolve the barbiturate completely. The clear, colorless, or nearly colorless solution so obtained was suitable for spectrophotometric determination of quantities of barbiturate less than 0.25 mg. Further dilution to an accurately measured volume was necessary when a larger amount was present in the eluate residue in order to secure a final concentration of 0.025 mg. per ml. or less.

Spectrophotometric determination of the barbiturate concentration of the 0.45 *N* sodium hydroxide solution obtained from the eluate was carried out in exactly the same manner as described for the standardization, using the differential technique described by Williams and Zak (5). The quantity of barbiturate present was then calculated by comparison of the spectrum so obtained with that of a standard solution of the barbiturate.

In order to evaluate the recovery of barbiturate from a Florisil column, the procedure was carried out using 10.0-ml. aliquots of the standard solutions. When the eluate residue was subsequently dissolved in 0.45 *N* sodium hydroxide to make 10.0 ml. of solution, the percentage of barbiturate recovered could then be calculated from the relationship

$$\frac{(A_{max} - A_{min})\ \text{eluate}}{(A_{max} - A_{min})\ \text{standard}} \times 100 = \text{per cent recovery}$$

The recovery of pentobarbital from pure aqueous solution (Table I) and from urine (Table II) is representative of the results obtained for all of the barbiturates studies. The actual absorbances as read from the differential curves, together with the percentage recovered using 10.0 ml. of the indicated concentrations of standard solutions are shown in Table I. A 10.0-ml. portion of each of the standard solutions was added to 10 ml. of drug-free urine to obtain the results listed in Table II. Duplicate determinations were made as an indication of the reliability of the method.

The separation of barbiturate from salicylate using the Florisil column technique was demonstrated by addition of an aliquot of a solution containing 10 mg. of salicylic acid to 10.0 ml. of standard barbiturate solution before extraction and passage of the chloroform extract through the adsorption column. The results of duplicate analyses carried out on phenobarbital are shown in Table III.

Table IV lists the average recoveries obtained by duplicate determinations on each of the four barbiturates investigated. In each case 10.0 ml. of a standard barbiturate solution and 20 mg. of salicylic acid were added to 10 ml. of drug-free urine prior to extraction and separation on a Florisil column.

TABLE II.—RECOVERY OF PENTOBARBITAL FROM URINE BY USE OF FLORISIL COLUMNS

Concn. Standard, mcg. per ml.	Added to 10 ml. Urine, mg.	Absorbance, $A_{max} - A_{min}$: Standard	Eluate	Recovered, mg.	Recovery, %
5.024	0.0502	0.197	0.199	0.0508	101.0
5.024	0.0502	0.197	0.196	0.0500	99.5
10.05	0.1005	0.395	0.390	0.0992	98.7
10.05	0.1005	0.395	0.392	0.0997	99.2
20.10	0.2010	0.776	0.767	0.1987	98.8
20.10	0.2010	0.776	0.771	0.1997	99.4

TABLE III.—RECOVERY OF PHENOBARBITAL FROM AQUEOUS SOLUTIONS CONTAINING 10 MG. OF SALICYLIC ACID BY USE OF FLORISIL COLUMNS

Concn. of Standard, mcg. per ml.	Added, mg.	Absorbance, $A_{max} - A_{min}$: Standard	Eluate	Recovered, mg.	Recovery, %
4.980	0.0498	0.194	0.193	0.0495	99.5
4.980	0.0498	0.194	0.192	0.0493	99.0
9.960	0.0996	0.394	0.397	0.1004	100.8
9.960	0.0996	0.394	0.394	0.0996	100.0
19.92	0.1992	0.793	0.789	0.1982	99.5
19.92	0.1992	0.793	0.799	0.2007	100.8

TABLE IV.—RECOVERY OF BARBITURATES FROM URINE CONTAINING 20 MG. OF SALICYLIC ACID BY USE OF FLORISIL COLUMNS

Barbiturate	Added, mg.	Recovered, mg.	Recovery, %
Amobarbital	0.0480	0.0473	98.5
	0.0960	0.0946	98.6
	0.1920	0.1913	99.7
	4.800	4.798	99.9
Pentobarbital	0.0502	0.0504	100.3
	0.1005	0.0995	99.0
	0.2010	0.1992	99.1
	5.024	5.008	99.7
Phenobarbital	0.0498	0.0501	100.5
	0.0996	0.0977	98.1
	0.1992	0.1977	99.3
	4.980	5.033	101.0
Secobarbital	0.0499	0.0499	100.0
	0.0999	0.1000	100.1
	0.1998	0.1978	99.0
	4.994	4.970	99.5

As a further test of the Florisil separation technique, the column procedure was applied to actual toxicological specimens. In a limited number of such cases in which sufficient urine containing barbiturate was available for experimental purposes, a 10.0-ml. sample of the urine was extracted and subjected to the Florisil column procedure in order to determine the concentration of barbiturate present. To a second 10.0-ml. portion of the specimen an aliquot of a standard barbiturate solution was added and the procedure was again carried out. The results of these analyses are shown in Table V. The barbiturate concentration before and after addition of a known quantity of a specific malonylurea derivative was then calculated from the standard spectrophotometric calibration curve for that particular compound. Thus, when phenobarbital was added, the amount of barbiturate originally present in the specimen was calculated as phenobarbital. In this way the amount of phenobarbital recovered could be determined by subtracting the number of milligrams originally present from the total number of milligrams recovered after addition of a known quantity of phenobarbital.

A ferric chloride test was performed to indicate the presence or absence of salicylate in each specimen. Urine samples B and C were large enough to permit separate determination of salicylate by the method of Williams and co-workers (6). In these samples the salicylate concentration was so high that the absorbance peak at 318 mμ could be used to estimate the salicylate even though barbiturate was also present.

DISCUSSION

A procedure for determination of barbiturates in urine specimens containing salicylate is described which involves the separation of barbiturate from salicylate by selective adsorption on Florisil. A chloroform extract obtained from urine is passed through a Florisil column. Upon subsequent elution with a 10% solution of anhydrous methanol in chloroform, salicylate as well as other chromogenic material normally present in urine extracts remains adsorbed, while barbiturate is recovered quantitatively in the eluate. Spectrophotometric determination of the barbiturate can then be carried out without interference from salicylate and urinary constituents which exhibit absorption in the ultraviolet region.

Selection of Adsorbent.—Of the several adsorbents investigated, only Florisil was found to give consistent and reproducible results. This magnesia-silica gel preparation has been successfully used for the chromatography of a wide variety of compounds which have otherwise proven difficult to separate. The 60/100 mesh grade may be easily and uniformly packed in a column which permits the solvent to flow at a fairly rapid rate. The material activated at 1200° F. or 500° F. was found to give equally satisfactory results. The complete retention of at least 20 mg. of salicylic acid on the adsorption column (Table IV), together with the purity of the barbiturate eluate obtained attest to the efficiency of Florisil as used in the procedure described in this study.

Selection of Solvents.—In order to facilitate extraction of barbiturate from urine samples acidified with hydrochloric acid, ether rather than chloroform was used. Troublesome emulsions usually encountered with chloroform were thus avoided, and the ether as well as the small amounts of dissolved water and hydrochloric acid present in the extract could be readily evaporated on a 70° water bath. Application of an ether extract containing dissolved water and hydrochloric acid to a Florisil column resulted in incomplete retention of salicylate and urinary chromogenic material but barbiturate was apparently not adsorbed and could be at least partially removed from the column by washing with ether. The removal of some water-soluble material from the Florisil which produced a yellow color in 0.45 *N* sodium hydroxide was also found to be due to the presence of water and hydrochloric acid. When an ether extract was evaporated to dryness to remove all traces of water and hydrochloric acid and a dry ether solution of the residue was applied to a Florisil column, barbiturate, salicylate, urinary pigments, and chromogens were completely adsorbed.

A number of different solvents were investigated in order to determine optimum conditions for retention of salicylate and urinary chromogens during elution of barbiturate from a Florisil column. A close parallel was found between the action of these solvents and their polarity. Although barbiturate was completely adsorbed from ether solution, the somewhat more polar solvents, chloroform and ethyl acetate, permitted small amounts of barbiturate to pass through the column when 50 ml. of either solvent were applied, while pigments and salicylate were retained. On application of acetone, urinary chromogenic material was removed. Increasing the polarity still further by use of ethanol or methanol resulted in partial elution of salicylate, which was accompanied by removal of most of the urinary pigments as well. It therefore became necessary to select a suitable solvent for elution of barbiturate which would not elute salicylate or urinary chromogenic material under practical analytical conditions. A 10% solution of anhydrous methanol in redistilled chloroform proved effective for this purpose. The addition of methanol to chloroform in this proportion produced a solvent of

TABLE V.—RECOVERY OF BARBITURATES ADDED TO TOXICOLOGICAL SPECIMENS OF URINE CONTAINING BARBITURATE BY USE OF FLORISIL COLUMNS

Urine Specimen →	A	B	C	D
Salicylate present, mg. %	0	9.2	10.8	[a]
Barbiturate present[b] in 10.0 ml. of urine, mg.	0.1141	0.1908	0.7071	0.1330
Barbiturate added to 10.0 ml. of urine, mg.	Phenobarbital 0.09960	Phenobarbital 0.1992	Phenobarbital 0.1992	Secobarbital 0.0999
Total barbiturate[b] recovered, mg.	0.2176	0.3927	0.9108	0.2354
Recovery of added barbiturate, %	103.9	101.3	102.3	102.5

[a] The presence of salicylate in urine specimen D was assumed on the basis of a strongly positive reaction to ferric chloride.
[b] In each case the amount of barbiturate was calculated in terms of the particular compound added as a recovery standard.

sufficient polarity to elute as much as 5 mg. of barbiturate (corresponding to a urinary concentration of 50 mg. % based on a 10-ml. sample) using only 25 ml. of eluant (Table IV).

The capacity of the adsorption column for retention of salicylate under the condition used for elution of barbiturate was determined by extracting aliquots of standard salicylic acid solutions and subjecting the extracts to the Florisil column procedure. Although the quantity of salicylate added to urine samples was limited to 20 mg. for recovery experiments on barbiturates (Table IV), it was found that as much as 25.0 mg. of salicylic acid could be retained successfully on the column provided that no more than 25.0 ml. of 10% methanol in chloroform was added in the elution step.

While urinary pigments as well as salicylate were not removed from a Florisil column by washing with chloroform, it was found that barbiturate was not completely retained on the column from chloroform solution, a small amount appearing in the effluent. For this reason the eluate obtained by treatment of the column with 10% methanol in chloroform was combined with the chloroform effluent. By use of these particular solvents, salicylate and interfering substances present in urine extracts were completely eliminated from the barbiturate eluates. The degree of purity of the eluates thus obtained made possible the quantitative recovery of as little as 0.05 mg. of barbiturate (Table IV), corresponding to a level of 0.5 mg. % in urine using a 10-ml. sample.

The technique herein described has been applied successfully in the isolation of barbiturates from a number of specimens of urine for toxicological purposes. That recovery of barbiturate by this method is quantitative from such specimens is evident from the data in Table V. It is to be noted that accurate determination of the relatively low barbiturate content of these urine samples was made possible by first removing the salicylate from the extract on a Florisil column. Chloroform extracts from blood and gastric contents have also been subjected to the Florisil purification process, resulting in solutions free of nonbarbiturate chromogenic substances and thus making possible the accurate determination of barbiturates by automatic differential ultraviolet spectrophotometry.

SUMMARY

A procedure for separation of barbiturates from urine specimens containing salicylate is described which is based on selective adsorption. Isolation of the barbiturate present in a chloroform extract is accomplished by means of an adsorption column from which the malonylurea derivative may be eluted conveniently while interfering substances are retained.

Salicylate, urine pigments, and other chromogenic materials which preclude spectrophotometric determination of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from chloroform solution by Florisil. Elution of the barbiturate with a small volume of 10% methanol in chloroform results in quantitative recovery of the drug in sufficient purity to permit spectrophotometric analysis.

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Radiochemical Purity of Tritium Exchange Labeled Barbital

By C. R. BOSHART†, G. K. W. YIM, and T. S. MIYA

Barbital (diethylmalonylurea) was isotopically labeled with tritium by the Wilzbach gas exposure procedure. The specific activity of the tritiated barbital was 266 mc./Gm. after removal of labile tritium. The following criteria of homogeneity were employed: recrystallization to constant specific activity using 2 solvent systems; column liquid-liquid partition and paper chromatography; and evidence for stability in a biological system. Final specific activity was 253 mc./Gm.

THE ADVANTAGES associated with the use of tritium as a tracer isotope prompted our studies with barbital (diethylmalonylurea) tritiated according to the Wilzbach gas exposure procedure (1). Our purpose was to determine radiochemical purity as well as biological stability of this exchange labeled tritiated compound. Criteria of homogeneity included recrystallization to constant specific activity using two solvent systems; column liquid-liquid partition and paper chromatography; and evidence for stability in a biological system.

EXPERIMENTAL

Analytical.—Radioactivity measurements were carried out using the Tri Carb Beta liquid scintillation spectrometer.[1] Five-dram vials made from glass of low potassium content were used to hold radioactive samples.[2] Aqueous solutions containing tritium radioactivity were incorporated into a xylene:dioxane:ethanol (5:5:3) system containing in each liter, 80 Gm. naphthalene, 5 Gm. 2,5-diphenyloxazole (PPO), and 0.1 Gm. 1,4-di[2-(5-phenyloxazolyl)] benzene (POPOP) (2). Toluene containing 0.3% PPO and 0.01% POPOP was used for counting radioactive samples miscible with it. Barbital was determined spectrophotometrically in 0.5 *N* sodium hydroxide at 255 mμ (3).

Tritiation, Exchange, and Recrystallization.—Barbital sodium was tritiated according to the Wilzbach (1) procedure by the New England Nuclear Co., Boston, Mass. The compound was exposed to 10 c. of carrier-free tritium gas for 2 weeks at room temperature and 0.39 Atm. pressure. The tritiated product was repeatedly equilibrated with water by the New England Nuclear Co. and in our laboratory to remove labile tritium. Specific activity of the free acid following the equilibration steps was 266 mc./Gm. The tritiated barbital was successively crystallized from ethanol-water, three times from toluene, and finally from water, yielding a product which retained approximately 96% of the radioactivity present after removal of labile tritium. Unlabeled barbital was added before final recrystallization from water, however, so that the actual specific activity was reduced to 166 mc./Gm.

Paper Chromatography.—Unlabeled barbital and barbital-t were chromatographed on Whatman No. 1 paper using *n*-butanol:5% ammonium hydroxide (1:1) (4). Radioactive areas were visualized autoradiographically. The papers were sprayed with 0.1 *N* aqueous silver nitrate:10% ammonium hydroxide (1:1) for detection of the barbital (5). A substance with very low specific activity and which was more polar than barbital was detected in the first recrystallization product. For all other cases, the darkening of the film was coincident with the barbital-t area and parallel to unlabeled barbital chromatographed concurrently.

Column Liquid-Liquid Partition Chromatography.—Barbital-t was chromatographed on a diatomaceous earth[3] column (6) using borate buffer 0.1 *M*, pH 9.2 as the internal phase and chloroform:butanol (93:7) as the external phase. Following the third recrystallization from toluene, barbital-t exhibited a homogeneous peak followed by a minor peak containing less than 5% of the radioactivity. The homogeneity of the major component was confirmed by analyzing the specific activities of fractions throughout the peak by the method of Baggett and Engel (7). The slope of specific activities for successive tubes through the peak was not significantly different from zero. After recrystallization from water, the barbital-t exhibited a single peak as illustrated in Fig. 1. The specific activity of six combined peak fractions was the same before and after column partition and the minor peak was no longer evident.

Biological Stability.—A suitable dilution of the final product was injected intravenously into a rabbit. Two hours later barbital was recovered from the serum by the method of Askevold and Løken (8). Table I shows the amount of barbital and radioactivity injected and recovered from the serum. Specific activities shown in the last column agree within 5%. This is within the range of experimental error associated with the analytical procedure.

DISCUSSION

Three types of evidence have been presented to demonstrate the degree of purity of exchange-labeled tritiated barbital. First, there was negligible loss of tritium radioactivity upon successive

Received May 19, 1961, from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.
Accepted for publication July 1, 1961.
† Public Health Service Research Fellow of the National Institute of Neurological Diseases and Blindness.
[1] Model 314-X, Packard Instrument Co., La Grange, Ill.
[2] Five-dram crystallite vial No. A-3305, T. C. Wheaton Co., Millville, N. J.
[3] Celite 545, Johns-Manville Products, New York, N. Y.

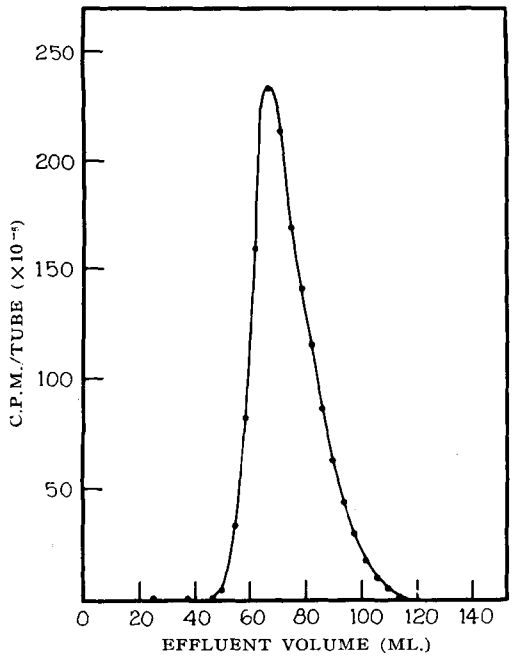


Fig. 1.—Celite column partition chromatogram of barbital-t.

recrystallization from toluene and water. Second, chromatographic methods indicated that a single substance, identical with barbital, constituted most of the detectable radioactivity. On paper chromatography of the first crystalline product, and in one column chromatogram of the third toluene recrystallization product, a minor radioactive component was present. The minor component of the column chromatogram constituted less than 5% of the radioactivity. After subsequent recrystallization from water, this secondary peak did not appear. Third, barbital-t lost little, if any, of its tritium after injection into an experimental animal and recovery after 2 hours. This latter observation nearly eliminates the possibility of the presence of high specific activity contaminants in barbital-t inasmuch as we would not expect, if contaminants were present, that their distribution, metabolic degradation, sojourn in the body, or chloroform extractability from serum would be identical with that of barbital. We have concluded from these results that a product with a high degree of radiochemical purity and stability suitable for pharmacologic studies was obtained. After removal of labile tritium by exchange with water, the barbital-t retained approximately 96% of the remaining isotope through recrystallization and chromatography. This is the highest yield yet reported for the Wilzbach exchange-labeling method. If the barbital-t had been subjected to the procedures outlined without dilution with unlabeled barbital, its specific activity would have been 253 mc./Gm. This represents the presence of approximately 1 tritium atom per 610 molecules of barbital. The results with this compound are unusual since there was little evidence for the presence in the labeled material initially of a high specific activity contaminant.

TABLE I.—BARBITAL AND RADIOACTIVITY INJECTED AND RECOVERED FROM RABBIT SERUM

Barbital-t	Radioactivity, C/M	Sample Weight, mcg.	Specific Activity C/M per mcg.
Administered to rabbit	2.04×10^9	6.22×10^5	3280
Isolated from rabbit serum[a]	1.125×10^7	3.6×10^3	3125

[a] Two hours following intravenous injection.

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Acid and Enzymic Hydrolysis of N-Butylformamide and Its Oral Inefficacy

By EDWARD R. GARRETT† and DENNIS J. WEBER

The kinetics of acid and enzymic hydrolysis of the parenterally pharmacologically active N-butylformamide have been studied by titrimetric and color assay procedures. It is expected that the half-life of the amide under the conditions of the human stomach would exceed 30 hours. The stability of the drug greatly exceeds any stomach retention time.

INITIAL recognition of the pharmacological efficacy of a drug often results from a screening program where the potential agent is parenterally administered. However, this mode of administration severely limits a drug's utility and marketability so that the product potential has to be evaluated with regard to oral efficacy. If a drug is parenterally effective but orally ineffective, a physical-chemical evaluation may possibly demonstrate the reason so that the drug may be properly chemically or pharmaceutically modified (1).

The compound, N-butylformamide, $HCNCH_2-CH_2CH_2CH_3$ (O double-bonded to C; H on N), was shown to have a selective effect on the avoidance behavior of rats similar to that of chlorpromazine when administered parenterally but not when administered orally (2). The purpose of this study was to predict the possible gastric stability of the material for a possible explanation of its oral inefficacy.

EXPERIMENTAL

Kinetics of Acid Hydrolysis.—Weighed amounts of the liquid N-butylformamide, synthesized by R. B. Moffett of these laboratories, were placed in thermally equilibrated 50-ml. volumetric flasks and brought up to volume with thermally equilibrated standard hydrochloric acid, immediately mixed, and maintained in the constant temperature bath. The detailed concentrations and temperatures are given in Table I. Five-milliliter aliquots of the hydrolyzing amide solutions were taken at planned intervals and quenched with sufficient 1 *M* sodium hydroxide so that the excess hydrochloric acid was almost neutralized. To the 6 ml. of the resultant solution was added 25 ml. dimethylsulfoxide, and the resultant mixed solution was cooled to room temperature. This solution was subsequently titrated with standard 0.1 *N* sodium hydroxide on the Precision-Dow recording titrator equipped with glass-saturated calomel electrodes. This solvent mixture gave two sharp inflections, the first one indicative of complete neutralization of the excess hydrochloric acid and the second one indicative of the complete neutralization of the formic acid produced by the acid hydrolysis.

Kinetics of Enzymic Hydrolysis.—Accurately weighed samples of N-butylformamide were diluted to 50 ml. with thermally equilibrated degrading solutions. There were three different degrading solutions used. One contained 0.4 *N* hydrochloric acid and 0.1% w/v pepsin at 40°; another contained 0.01 *N* hydrochloric acid and 0.1% w/v pepsin at 40°; and the third contained a Clark and Lubbs buffer (3) of pH 7.9 at 40° and contained 0.1% w/v trypsin. The pepsin and trypsin were obtained from the Worthington Biochemical Corp.

One-milliliter aliquots were taken at recorded time intervals and their N-butylamine content determined by the method evaluated by Forist and Judy (4) of these laboratories. The spectrophotometric procedure for the determination of amines based on reaction with bromcresol purple in chloroform (5) was applicable to the determination of N-butylamine in chloroform (4). Under the conditions of extraction (0.1 *N* sodium hydroxide) production of butylamine from intact butylformamide was less than 2% over a period of 15 minutes and indicated adequate stability of the amide. Beer's law was followed for butylamine over the range 10^{-4} to 10^{-3} *M*, the concentration range of the samples. Determination of standard butylamine samples gave a mean recovery ± standard deviation of 99.1 ± 3.5% (4). The presence of enzyme was shown not to interfere with the assay.

CALCULATIONS AND RESULTS

The basic expression for the hydrolysis kinetics is

$$d(A - X)/dt = -k[A - X] = -k_{H^+}[H^+][A - X] \quad \text{(Eq. 1)}$$

where A is the initial concentration of the N-butylformamide, X is the concentration of formed formic acid at time t, and k is the pseudo first-order rate constant obtained from the slope of the first-order plot of the integrated expression

$$\log [A - X] = -kt/2.303 + \log [A] \quad \text{(Eq. 2)}$$

Typical plots are given in Fig. 1. The conditions, pseudo first-order and actual second-order rate constants, are given in Table I.

The Arrhenius plot of the logarithm of the rate constant, k_{H^+} in L/M/sec., against the reciprocal of the absolute temperature as per

$$\log k = -(\Delta H_a/2.303R)(1/T) + \log P \quad \text{(Eq. 3)}$$

is given in Fig. 2. The heat of activation is 19.8 Kcal./mole and the log P value is 9.16.

Apparent rate constants for the hydrolysis of N-

Received September 6, 1961, from the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication September 25, 1961.
† Present address: University of Florida, College of Pharmacy, Gainesville.

TABLE I.—KINETICS OF ACID HYDROLYSIS OF N-BUTYLFORMAMIDE

Temp., °C.	[HCl]	[N-Butylformamide]	10^6k (sec.$^{-1}$)	$t_{1/2}$, hr.	$10^5k/[HCl] = 10^5 k_{H^+}(L/M/sec.)$
30.0	0.0993	0.0400	0.747	258	0.750
30.0	0.1988	0.0421	1.40	138	0.703
30.0	0.3989	0.0388	2.94	65.6	0.738
39.0	0.4045	0.0331	8.47	22.8	2.08
50.6	0.0994	0.0449	6.69	28.7	6.74
50.5	0.1988	0.0182	14.7	13.1	7.39
50.2	0.1988	0.0182	12.6	15.3	6.34
51.0	0.4045	0.0288	23.1	8.34	5.70
60.0	0.4045	0.0355	63.6	3.02	15.7
70.0	0.0993	0.0210	27.9	6.80	28.1
70.8	0.1988	0.0192	81.9	2.35	41.1
69.6	0.3989	0.0196	120	1.59	30.2

TABLE II.—DATA FROM SUPPOSED ENZYMATIC HYDROLYSIS OF N-BUTYLFORMAMIDE AT 40°

[H$^+$]	w/v % Pepsin	w/v % Trypsin	[*N*-Butylformamide]	10^6k (sec.$^{-1}$)	$t_{1/2}$, hr.	$10^5k/[H^+] = 10^5 k_{H^+}(L/M/sec.)$
0.4117	0.1	..	1.10×10^{-3}	8.60	22.3	2.09
0.0100	0.1	..	1.68×10^{-3}	0.0524	3670	0.524
$10^{-7.9}$	..	0.1	1.07×10^{-3}	*ca.* 0.00	>3670	*ca.* 0.0

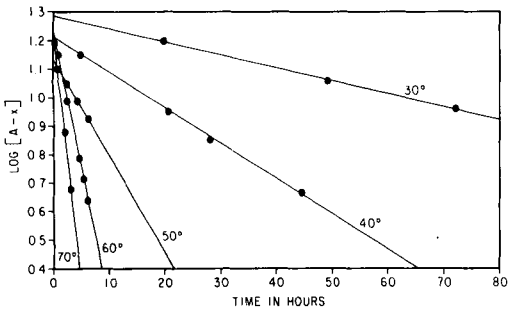


Fig. 1.—Pseudo first-order plots of the hydrolysis of N-butylformamide in 0.4 *N* hydrochloric acid. The temperatures and the initial molarities of *N*-butylformamide were: 30°, 0.0388; 40°, 0.0331; 50°, 0.0288; 60°, 0.0355; and 70°, 0.0196.

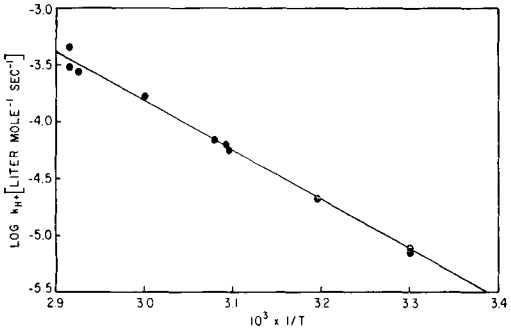


Fig. 2.—Arrhenius plot of the bimolecular rate constant for the acid hydrolysis of N-butylformamide.

butylformamide in the presence of the enzymes trypsin and pepsin are given in Table II. A specific example of a pseudo first-order rate plot is given in Fig. 3. The line is drawn on the basis of the acid hydrolysis equations evaluated in Eq. 2 and shows no enhancement of rate by the presence of the enzyme pepsin. The pH 2 solution of pepsin, a better approximation of stomach conditions, also demonstrated a hydrolysis rate not inconsistent with simple acid hydrolysis. The experiment using trypsin at its optimum pH value also showed no significant enzymic effect.

DISCUSSION

Kinetic analysis of the enzymatic degradation of N-butylformamide showed that the oral inefficacy of the drug cannot be attributed to gastrointestinal degradation by the enzymes, pepsin or trypsin, at their optimum pH values, 2 and 7.8, respectively. Since the half-life of N-butylformamide at 39° in 0.4 *N* hydrochloric acid is 22.8 hours, the inefficacy of oral administration where parenteral administration is effective cannot be attributed to acid degradation of the compound in the gastrointestinal tract. This large half-life proves that the rates of acid degradation are grossly less than any stomach retention time.

This work indicates that the lack of oral activity is not due to enzymatic or solution degradation in the gastrointestinal tract. An alternative explanation is that the drug is orally inactive due to its inability to pass through the lipid-like gastrointestinal mucosa. This is consistent with its high insolubility in fat solvents (6).

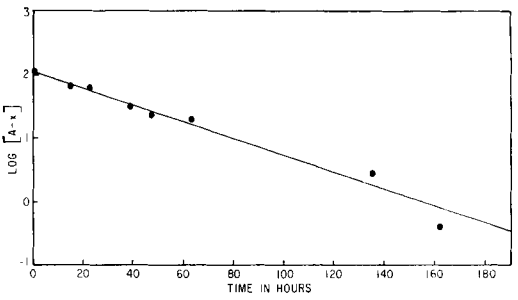


Fig. 3.—Pseudo first-order plot of the hydrolysis of N-butylformamide in the presence of 0.1% w/v pepsin at 40° and 0.411 *M* hydrochloric acid. The drawn line is expected from acid hydrolysis alone.

The heats of activation for the acid hydrolysis of various unsubstituted acylamides listed in the literature (7) are all in the range 19–21 Kcal./mole. These amides include formamide, acetamide, benzamide, propionamide, and butyramide. However, all of these compounds hydrolyzed at much faster rates; for example, formamide has a half-life at 42° in 0.1 *N* hydrochloric acid of 2.2 hours, at 25° of 7.0 hours (7). Apparently substitution on the nitrogen of the amide markedly slows the rate of hydrolysis.

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(6) Brodie, B. B., and Hogben, C. A. M., *J. Pharm. London*, **9**, 345(1957); and references therein.
(7) "Tables of Chemical Kinetics," U. S. Dept. of Commerce, National Bureau of Standards, Washington, D. C., Circular 510.

Preparation of Microcrystalline Progesterone Using Ultrasound

By JOSEPH R. PRINCIPE† and DONALD M. SKAUEN

Microscopic crystals of progesterone were prepared by insonating saturated solutions of the hormone. Suspensions were made of the crystalline precipitates in 1% tyloxapol solution. Individual crystals were measured by projecting photographs on a calibrated screen.

THE DESIRABILITY of obtaining a chemical compound of uniform microscopic size is considered to be of importance to the pharmaceutical industry, particularly in the preparation of suspensions.

The usual methods of preparing suspensions for injection involve size reduction by milling or crystallization from various solvents to produce crystals of microscopic size and uniformity.

This investigation was undertaken to ascertain the suitability of employing ultrasonic energy in the production of microcrystalline progesterone.

EXPERIMENTAL

Saturated solutions of progesterone in solvent mixtures of varying proportions of ethyl alcohol and ethylene glycol at different temperatures were prepared. This solvent was selected because of good solvent action and because viscosity could be varied without the use of a secondary agent.

One-milliliter samples of the supernatant liquid from the saturated solutions were transferred to small hard-glass test tubes, stoppered, and immediately insonated.[1] The contents of the sound chamber was kept at approximately 20° by means of a cold water coil. The effect of length of time of insonation and the effect of power were explored.

Immediately after insonation, the test tubes containing the resulting precipitates were centrifuged, the supernatant discarded, and the crystals allowed to dry before resuspension in a 1% aqueous tyloxapol solution.

Photomicrographs of the uniformly distributed suspended material were made and examined for particle size by a projection technique.

Table I shows typical data obtained by insonating saturated solutions prepared at 55°.

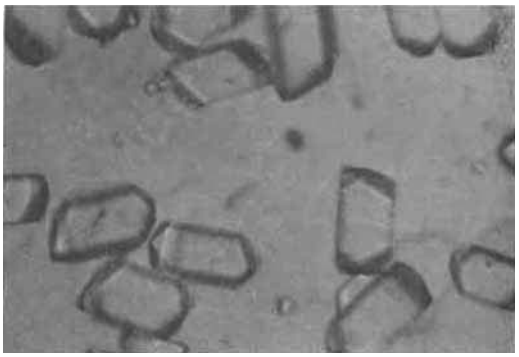


Fig. 1.—Prepared progesterone crystals. Solvent, ethyl alcohol U.S.P., 25 parts, ethylene glycol, 75 parts; insonation time, 5 sec.; arithmetic mean length (μ), 47.3; saturated solution prepared at 55°; plate power, 50 ma.; S.D., 9.4.

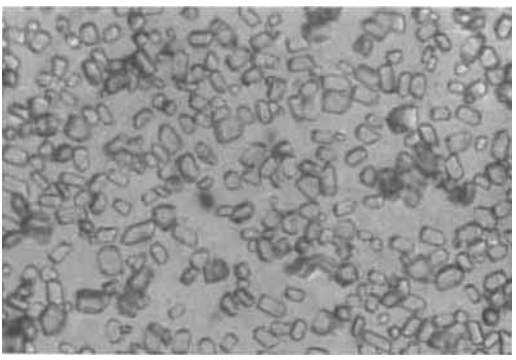


Fig. 2.—Prepared progesterone crystals. Solvent, ethyl alcohol U.S.P., 25 parts, ethylene glycol, 75 parts; insonation time, 5 seconds; arithmetic mean length (μ), 11.0; saturated solution prepared at 55°; plate power, 100 ma.; S.D., 3.3.

Received May 27, 1961, from the University of Connecticut, School of Pharmacy, Storrs.
Accepted for publication December 7, 1961.
† Research Pharmacist, Colgate Pharmaceutical Laboratories, 1345 Easton Ave., New Brunswick, N. J.
[1] Model G-3, General Electric Ultrasonic Generator.

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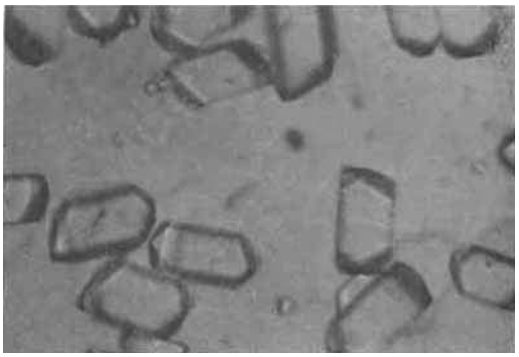


Fig. 1.—Prepared progesterone crystals. Solvent, ethyl alcohol U.S.P., 25 parts, ethylene glycol, 75 parts; insonation time, 5 sec.; arithmetic mean length (μ), 47.3; saturated solution prepared at 55°; plate power, 50 ma.; S.D., 9.4.

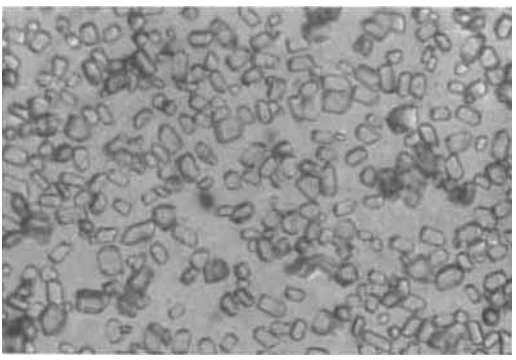


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[1] Model G-3, General Electric Ultrasonic Generator.

TABLE I.—PARTICLE SIZE OF PREPARED PROGESTERONE CRYSTALS[a]

Insonation Time / Plate Power, ma.	5 sec. Arithmetic Mean, μ	5 sec. S.D.	15 sec. Arithmetic Mean, μ	15 sec. S.D.	25 sec. Arithmetic Mean, μ	25 sec. S.D.
50	47.3	9.4	23.7	12.2	18.6	9.4
75	16.9	7.5	9.3	4.4	13.5	4.9
100	11.0	3.3	7.8	4.4	8.8	4.1
125	16.9	4.7	9.5	4.5	9.9	4.5
150	14.5	7.1	10.2	8.2	7.3	3.1

[a] Solvent, ethyl alcohol U.S.P., 25 parts, ethylene glycol, 75 parts; insonation time, 5 seconds; saturated solution prepared at 55°.

Figure 1 shows crystals obtained under the conditions specified in Table I, at a plate power of 50 ma. and 5 seconds exposure time. Figure 2 shows crystals exposed for 5 seconds at a plate power of 100 ma.

SUMMARY

1. Microscopic crystals of progesterone were prepared by insonating saturated solutions of the hormone.

2. The results indicated that: (*a*) in general, an increase in plate power from 50 to 100 ma. results in smaller crystals; (*b*) the solvent system 25% ethyl alcohol–75% ethylene glycol appears to favor smaller and more uniform crystal size; (c) the temperature at which the saturated solution is prepared seems to affect crystal size; (*d*) length of time of insonation appears to have minimal influence on crystal size.

3. Crystals prepared under insonation conditions were smaller and more uniform than controls prepared without insonation.

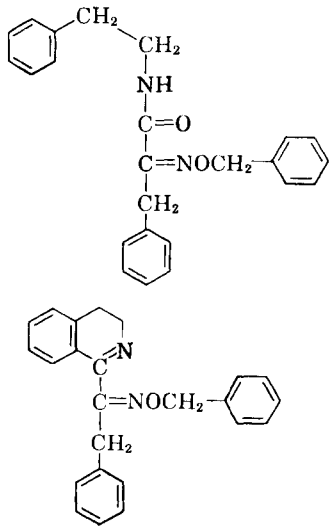
Amides of α-Alkyloximino Acids

By WALTER H. HARTUNG† and DOMINICK A. COVIELLO‡

N-β-Phenethyl amides of α-benzyloximino acids were prepared as intermediates for an attempted cyclization to the corresponding 3,4-dihydroisoquinoline derivatives.

THE ISOQUINOLINE nucleus is found in a large number of medicinally active compounds, both natural and synthetic. With this and the fact that little is known about the biological activity of the alkyloximino group in mind, it was thought that if a substituent containing the alkyloximino group could be introduced into the isoquinoline nucleus a compound of biological interest might result. At the same time, there was also the opportunity to explore further the chemical stability and limitations of the group under various conditions of reaction.

The plan was to prepare N-β-phenethyl amides of α-benzyloximinopropionic acids with the hope that they could be cyclized under the conditions of the Bischler-Napieralski reaction (1) to obtain new 3,4-dihydroisoquinoline derivatives containing the alkyloximino group. These presumably could be dehydrogenated to yield isoquinolines. For example, N-β-phenethyl-β-phenyl-α-benzyloximinopropionamide would yield 1-(1-benzyloximino-2-phenylethyl) 3,4-dihydroisoquinoline.



The amides prepared in this investigation are listed in Table I.

Cyclization was attempted by refluxing the amides

Received November 14, 1961, from the Medical College of Virginia, School of Pharmacy, Richmond.
Accepted for publication December 5, 1961.
† Deceased.
‡ Present address: University of Illinois, College of Pharmacy, 833 South Wood St., Chicago 12.

TABLE I.—PARTICLE SIZE OF PREPARED PROGESTERONE CRYSTALS[a]

Insonation Time / Plate Power, ma.	5 sec. Arithmetic Mean, μ	5 sec. S.D.	15 sec. Arithmetic Mean, μ	15 sec. S.D.	25 sec. Arithmetic Mean, μ	25 sec. S.D.
50	47.3	9.4	23.7	12.2	18.6	9.4
75	16.9	7.5	9.3	4.4	13.5	4.9
100	11.0	3.3	7.8	4.4	8.8	4.1
125	16.9	4.7	9.5	4.5	9.9	4.5
150	14.5	7.1	10.2	8.2	7.3	3.1

[a] Solvent, ethyl alcohol U.S.P., 25 parts, ethylene glycol, 75 parts; insonation time, 5 seconds; saturated solution prepared at 55°.

Figure 1 shows crystals obtained under the conditions specified in Table I, at a plate power of 50 ma. and 5 seconds exposure time. Figure 2 shows crystals exposed for 5 seconds at a plate power of 100 ma.

SUMMARY

1. Microscopic crystals of progesterone were prepared by insonating saturated solutions of the hormone.

2. The results indicated that: (*a*) in general, an increase in plate power from 50 to 100 ma. results in smaller crystals; (*b*) the solvent system 25% ethyl alcohol–75% ethylene glycol appears to favor smaller and more uniform crystal size; (c) the temperature at which the saturated solution is prepared seems to affect crystal size; (*d*) length of time of insonation appears to have minimal influence on crystal size.

3. Crystals prepared under insonation conditions were smaller and more uniform than controls prepared without insonation.

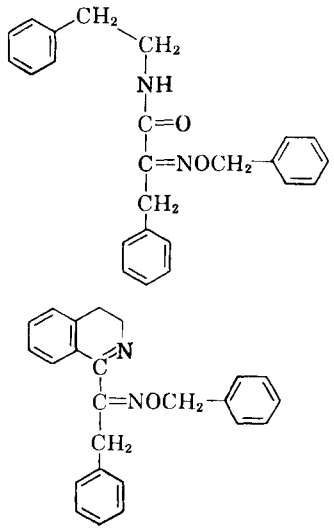
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N-β-Phenethyl-β-phenyl-α-benzyloximinopropionamide	54–56	55.3	7.53	7.52[a] 7.54
N-β-(3,4-Dimethoxyphenethyl)-β-phenyl-α-benzyloximinopropionamide	46–48	40.3	6.48	6.47 6.35
N-β-(3,4-Methylenedioxyphenylethyl)-β-phenyl-α-benzyloximinopropionamide	53–54	33.7	6.71	6.67 6.52
N-β-Phenethyl-α-benzyloximinopropionamide	41–43	[b]	9.49	9.38 9.22

[a] *Anal.*—Calcd. for $C_{24}H_{24}N_2O_2$: C, 77.4; H, 6.45; N, 7.53. Found: C, 77.3, 77.4; H, 6.24, 6.33; N, 7.3. Analyses done by Drs. Weiler and Strauss, Oxford, England. [b] No yield was calculated on this compound because an attempt was made to distil a portion since it showed no inclination to crystallize by the usual methods. The remaining portion, standing at room temperature for several weeks, crystallized. The portion which distilled at 184–195°/0.2 mm. did not crystallize even after standing for several months.

in toluene in the presence of phosphorus oxychloride and phosphorus pentoxide. The experiment was repeated with *p*-xylene solvent and without solvent. In another experiment, the amide was allowed to stand at room temperature for one week in phosphorus oxychloride. Another experiment at room temperature employed phosphorus pentoxide with the phosphorus oxychloride. In no case was a product isolated which had amine properties.

In light of this, an attempt was made to effect cyclization with polyphosphoric acid according to the procedure of Snyder and Werber (2). By this method the amine moiety was recovered and characterized as the picrate. From this it was assumed that the unchanged amide was hydrolyzed during decomposition of the hot reagent with ice. This was borne out by the fact that if the reaction mixture was allowed to cool before decomposition of the reagent, nothing could be isolated that had amine properties.

Experiments using polyphosphoric acid were run varying reaction time from 30 seconds to one and one-half hours at temperatures ranging from 50 to 145°, and at room temperature for one week without success.

EXPERIMENTAL

Amides were best prepared by the procedure of Vaughan and Osato (3). The products appeared at first to be intractable oils, but a pure product was ultimately obtained by taking up the oil in methanol and adding hexane until precipitation was imminent. After 2 days to a week in the freezing compartment of the refrigerator, a solid formed which was crystallized from methanol.

REFERENCES

(1) Bischler, A., and Napieralski, B., *Ber.*, **26**, 1903(1893).
(2) Snyder, H. R., and Werber, F. X., *J. Am. Chem. Soc.*, **72**, 2962(1950).
(3) Vaughan, J. R., Jr., and Osato, R. L., *ibid.*, **73**, 5553 (1951).

Use of an Approximate Dielectric Constant in Solubility Studies

By WILLIS E. MOORE

THE DATA published by Autian and Udani (1) on the solubility of secobarbital have been further evaluated in terms of solubility in relation to the approximate dielectric constant (A.D.C.) of the solvent(s) by the techniques proposed by Moore (2).

This additional analysis is noted here to further illustrate the utility of using an A.D.C. in solubility studies.

The binary systems in Autian and Udani's work (their Fig. 2) were examined. Only glycerin showed a linear relationship between A.D.C. and solubility [log A.D.C. was plotted *vs.* (concentration)$^{1/2}$ of solute]. Alcohol, propylene glycol, and PEG 400 showed varying degrees of positive deviation.

The ternary systems (their Fig. 3) show linear portions in all three systems when plotted as above. The slopes differed, since solubility was different, but the intercepts were identical at a log A.D.C. value equal to an A.D.C. of 88 (see Fig. 1). This predicts a zero solubility of secobarbital in a mixed solvent with an A.D.C. of 88. This value could, of course, be meaningless. Further investigation of this relationship is required for better understanding.

Figure 1 further shows that an inconsistency appears. For example, at an A.D.C. value of 59.5 (log A.D.C. = 1.775) the solubilities of secobarbital are predicted as in Table I.

Why the solubility should vary at a constant A.D.C. but with different solvent systems cannot be explained precisely by this empirically established relationship. It is recognized that the dielectric

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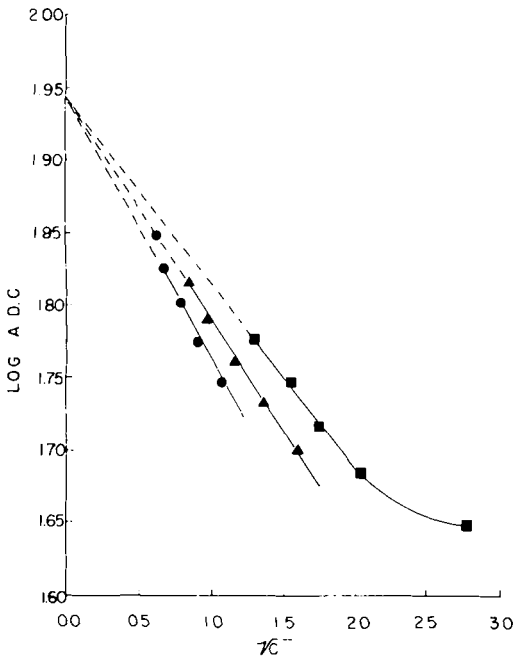


Fig. 1.—Relation of log A.D.C. to (concentration)$^{1/2}$. ●, 10% alcohol; ▲, 20% alcohol; ■, 30% alcohol.

constant of a solvent(s) system is only one factor, among many, that account for solubility.

The relationship of the apparent pKa values (their Fig. 7) for secobarbital and the A.D.C. of the solvent blend was also examined (apparent pKa was plotted *vs.* log A.D.C.). Figure 2 shows that the glycerin solvent blends were the only blends to exhibit a linear relationship. Alcohol and propylene glycol show linear portions but with positive deviations at low levels of the respective semipolar solvent. The distinctly different behavior of the PEG 400 blends is evident.

The mechanism of dissolution in aqueous blends of alcohol, propylene glycol, and glycerin may be the same (possibly solute-solvent interaction)

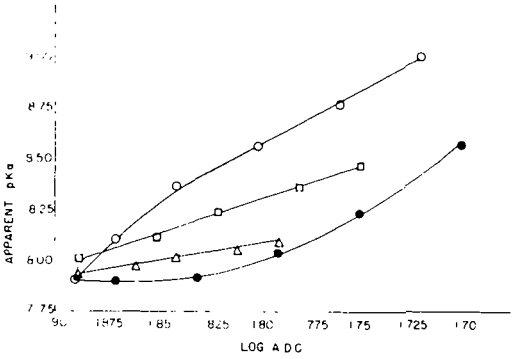


Fig. 2.—Effect of A.D.C. on pKa of secobarbital. ○, Alcohol; □, propylene glycol; △, glycerin; ●, PEG 400.

TABLE I.—PREDICTED SOLUBILITIES OF SECOBARBITAL

% Alcohol	% Glycerin	% Water[a]	$\sqrt{C}$	Concentration of Secobarbital, %
10	40.0	50.0	0.94	0.88
20	25.5[a]	54.5	1.10	1.21
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[a] Estimated.

differing only in degree. The adverse effect (i.e., lowering solubility) of water on the solvent power of the "good" solvents could be due to water competing with the solute for intermolecular H-bonding with the "good" solvent rather than a polarizing (i.e., dielectric) effect.

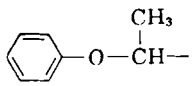
Additional experimental work such as examining the binary systems of glycerin with the other glycols and alcohol and conductance measurements of the entire series would provide further information, particularly since PEG 400 has, relatively, such a depressant effect on the ionization of secobarbital at concentrations above 40%.

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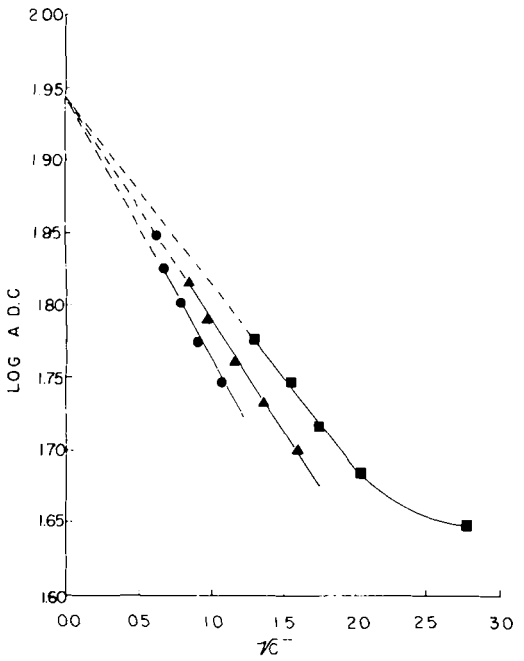


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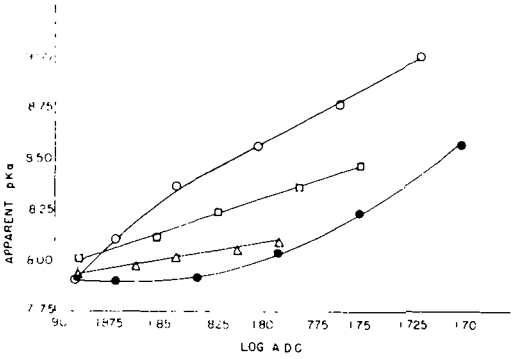


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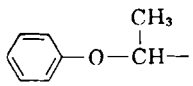
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Gas Chromatography of Sympathomimetic Amines

Sir:

Methods for the separation and identification of microquantities of sympathomimetic amines are of great importance for work in pharmacy, pharmacology, and toxicology. Although the literature in this field is extensive, there is still a need for a simple and rapid method that permits positive identification and lends itself to quantitative analysis.

This communication reports the separation and identification of 11 sympathomimetic amines by gas-liquid chromatography at temperatures ranging from 104° for the nonphenolic to 135° for the phenolic amines. A Barber Colman model 15 gas chromatograph equipped with an argon ionization detector has been used for this work. A suitable column is prepared from Gas-Chrom P, 100 to 140-mesh, coated with about 1% of silicone rubber SE-30. A typical sample is 1.0 μl. of a solution containing 0.5 to 1.0% of each amine. A mixture of six commonly used nonphenolic amines dissolved in chloroform gives five distinct peaks, ephedrine and pseudoephedrine being eluted together (Fig. 1). However, the two latter amines can be separated by using acetone as the solvent. Pseudoephedrine reacts relatively rapidly with acetone to produce an addition product, whereas the reaction between ephedrine and acetone is very slow.

Several sympathomimetic amines react with ketones. The reaction products produce sharp, symmetrical peaks, suitable for quantitative estimation. The compounds are readily identified on the basis of the relative retention values for the free amines and their ketone derivatives.

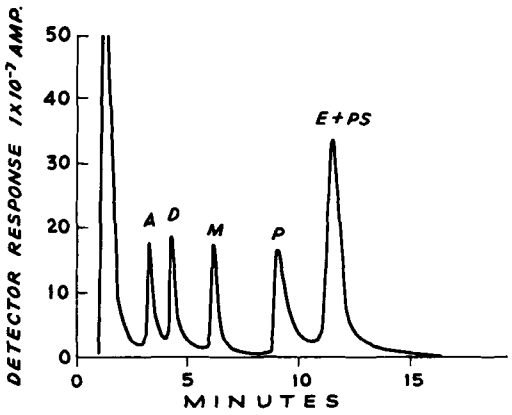


Fig. 1.—Gas chromatogram of six sympathomimetic amines on silicone rubber SE-30, 1.15%. Temp., 104°; column, 8 feet long, 3 mm. i.d.; pressure, 20 p.s.i.; flow rate, 22.5 ml./min. A, amphetamine; D, methamphetamine; M, mephentermine; P, phenylpropanolamine; E, ephedrine; PS, pseudoephedrine.

A complete report will be published shortly.

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Occurrence of Psilocin in *Psilocybe baeocystis*

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Recently, several dried carpophores of *P. baeocystis*, collected on October 30, 1960 from a lawn in Portland by C. Goetz, were made available to us through the courtesy of Mrs. H. J. Oswald. The identity of the specimens was verified by Prof. D. E. Stuntz, Department of Botany, University of Washington.

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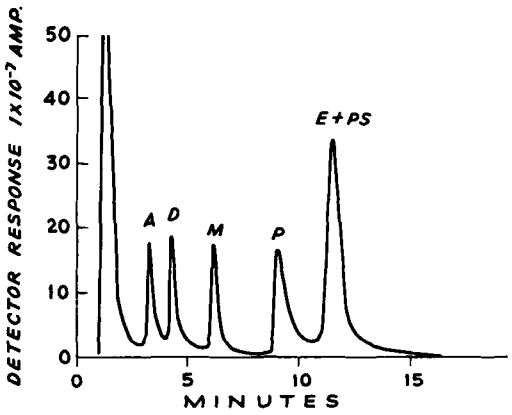


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examined chromatographically, essentially as described by Tyler (3). Solvent systems employed included water-saturated *n*-butanol, *n*-butanol:acetic acid:water (4:1:5), and *n*-propanol:1 *N* ammonia (5:1). In all three of these systems, a compound was detected by spraying with 2% *p*-dimethylaminobenzaldehyde in 1 *N* hydrochloric acid (blue-violet color) and with Pauly's reagent (orange-red color). It had an R_f value identical with reference psilocin and did not separate from that compound when spotted in admixture. Tryptophan was also identified on the chromatograms by its characteristic R_f value and the color formed with the *p*-dimethylaminobenzaldehyde reagent, but psilocybin could not be detected.

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New sections in this edition cover emission spectroscopy, automation, gas chromatography, and the theory and technique of the urea and creatinine clearance tests. The revised and expanded text is intended to make the book more suitable for use in teaching analytical clinical chemistry. Author and subject indexes are included.

Medicinal Chemistry. Vol. 5. Edited by Walter H. Hartung. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1961. vi + 432 pp. 14.5 × 23 cm. Price $18.

Volume five of this monograph series covers Anticonvulsant drugs and Bis(4-aminophenyl) sulfone and related compounds in tuberculosis and leprosy. The style and format of the earlier volumes are continued, and the objective to include in each chapter or monograph references to all the compounds that have been tested for a particular type of pharmacological activity still maintains. The extent of the work with anticonvulsants is indicated by the 1,113 references to publications and 16 pages of patents grouped according to chemical types. A general index is appended.

Structural Forms of Anesthetic Compounds. By Hugh S. Mathewson. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1961. xvi + 223 pp. 15 × 22.5 cm. Price $6.75

Intended as an introductory text, this book is based upon seminars on anesthetic drugs. The relation of structure-activity principles to the pharmacologic properties of drugs in clinical use by anesthesiologists is the frame upon which the subject is developed. An index is appended.

Progesterone and the Defence Mechanism of Pregnancy. Edited by G. E. W. Wolstenholme and Margaret P. Cameron. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1961. viii + 108 pp. 12 × 18.5 cm.

A compilation of the reports by the Ciba Foundation Study Group No. 9 in February 1961. A general index is included.

Biological Activity of the Leucocyte. Edited by G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1961. viii × 120 pp. 12 × 18.5 cm.

A compilation of the reports by the Ciba Foundation Study Group No. 10 in March 1961. A general index is included.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

May 1962 volume 51, number 5

Review Article

Percutaneous Absorption

By MARTIN BARR

THE STUDY of the passage of medicinal substances through the skin offers a great challenge to research workers in the fields of pharmacy and dermatology. Although dermatological vehicles themselves may not penetrate the skin to any extent nor actually carry the medicament through the epidermal barrier, it is known that the clinical effectiveness of a drug may vary when it is incorporated in different vehicles (1). The choice of an optimum vehicle for a particular medicament depends on the physical and chemical properties of the drug alone and in the vehicle as well as the nature of the skin condition being treated. Much information has been gained on this subject in the last few years but much more is needed in order to treat the various skin disorders encountered with maximum effectiveness.

Intelligent formulation of dermatological preparations depends on a thorough understanding of percutaneous absorption. Although recent studies have led to a better understanding of this phenomenon, there are many uncovered facets concerning its mechanism. This paper serves as a review of studies made in the field of percutaneous absorption. Space limitations make it impossible to summarize every work in the field but an attempt has been made to review those which appear to be most significant to its understanding.

Before going further, it is appropriate to define the term "percutaneous absorption." Rothman (2) defines percutaneous absorption as the penetration of substances from the outside into the skin and through the skin into the blood stream. As the literature in this field is studied, it is found that many terms have been employed: namely, absorption, sorption, persorption, permeation, and penetration. As Blank (3) has stated, it is actually not necessary to define the term "percutaneous absorption" with great accuracy but it is of greater importance to know how molecules move into the skin from a unit area of cutaneous surface in a unit of time, and where these molecules go after leaving the skin surface.

MECHANISM OF PERCUTANEOUS ABSORPTION

The modern concepts of percutaneous absorption are relatively new, especially when compared to the long history of preparations used for application to the skin. The earliest records of the use of fats and oils as vehicles for cosmetics and medicaments date back to the early records of Babylonian and Egyptian medicine (4). Broadly sketching the historical views and experimentations, one finds that the subject may be divided into three periods: (*a*) the period antedating 1877 and covering 100 years, during which much of the evidence was accepted as indicating that a large number of substances, chiefly gases and volatile substances, penetrated the skin freely; (*b*) the period extending from 1877 until 1900, during which Fleischer's school of thought held forth, in which Fleischer and his collaborators concluded from their experiments, and a critical appraisal of the older observations, that the skin of man and higher animals was absolutely impermeable to all substances (5); and (*c*) the period from 1900 to the present, the first few years of which saw the decline of the absolute impermeability theory, chiefly under the impetus of Schwenkenbecher's work (6).

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Despite the voluminous literature in the field, there is as yet very little agreement on the basic mechanism which is largely responsible for percutaneous absorption through intact skin. One group of investigators is of the opinion that the major role of entry for medicaments is directly through the intact epidermis (transepidermal route). Another group believes that most absorption takes place through the appendages, either through the pilosebaceous apparatus or the spiral sweat glands. There is evidence that indicates it is likely that all these avenues take part in the transfer of substances through the skin (7). No attempt will be made in this review to resolve this controversy. It is very likely that a specific substance may prefer one avenue to the other, depending on the many factors which will be discussed later in this paper.

Before reviewing the work that has been done on the mechanism of percutaneous absorption, it is appropriate to consider briefly the anatomical zones a penetrating substance meets in transit from the skin surface to the blood vessels (Fig. 1). In order, it encounters a surface film of emulsified lipids, the stratum corneum (horny layer), the barrier, the stratum germinativum (living epidermis), and finally the dermis (true skin) and blood vessels. Other avenues of penetration through the skin are shown diagrammatically in Fig. 2.

Transepidermal Absorption.—Mali (8) states that the pathway through the epidermis is much more likely the main avenue of penetration for substances than are the sebaceous glands or sweat glands, simply because the epidermis presents a surface area 100 or 1,000 times greater than the other two.

Various anatomical zones of the skin behave differently with regard to transepidermal absorption (9). The film on the surface of the skin is composed of sebum, sweat, and desquamating horny layer, and has a complex chemical composition. The character and extent of its interaction with the penetrating agent are unpredictable. This surface film is discontinuous and offers relatively little resistance to a penetrating molecule (9).

The horny layer, which is 20 to 40 μ in thickness, is composed largely of keratin, a sulfhydryl containing protein which adsorbs a large amount of water and other polar compounds. It also contains surface lipids which may spread along the channel walls between cells and adsorb lipid-soluble material. In other words, the horny layer may act as a sponge, becoming a reservoir for the penetrating agent and maintaining a maximum concentration gradient just above the barrier, thus possibly hindering penetration. This is especially true for ions and dyes of low molecular weight. Blank and Gould (10), using an autoradiographic technique, have shown that ionic surfactants tagged with S^{35} are bound by the horny layer and often do not penetrate beyond the orifice of the hair follicle (9).

The barrier zone was originally demonstrated by Rein (11) who reported that it was located between the horny layer and granular layer underlying it and that it was an electronegatively charged barrier which repelled anions and attracted and held cations for further penetration. Rothman (12) reported that the barrier layer checked the transfer of water across the skin. Blank (13) showed graphically that the barrier to water transfer in skin is a layer a few microns thick at the base of the stratum corneum. He used the plastic tape stripping method of Wolf (14) and Pinkus (15) to remove the horny layer gradually. He found that the diffusion of water through skin remained low until the base of the horny layer had been stripped away; thereafter the permeability to water rose sharply. In 1951 Szakall (16) reported that after the horny layer had been stripped off with tape, the entire barrier could be removed on Tesa tape in a single stripping. Subsequently he recovered the barrier layer by dissolving the tape in petroleum ether. Mali (8) isolated the barrier utilizing another method in 1955.

The barrier layer is 10 μ thick and prevents the penetration of molecules having molecular weights greater than 200 or 300 (17); yet the diameter of the pores in the material in the intercellular spaces in the barrier is much larger than the largest penetrating molecule. Thus, the restraining force must be the molecular interaction between penetrant and pore contents. If the substance has a high electrostatic charge, e.g., ions, the attraction is so great that no penetration occurs. If the substance has a water-lipid partition coefficient of about 1.0, it has the highest permeability; the barrier must then have both polar and nonpolar groups in the pore contents (9).

The few molecules which do penetrate the barrier layer are either bound by the living epidermis or the dermis, or carried away by the tissue fluids to the lymphatic and blood vessels. The electron-dense zone which is present at the junction between the epidermis and the dermis offers little resistance to the penetrating molecules (9).

Blank, Griesemer, and Gould (18) have pub-

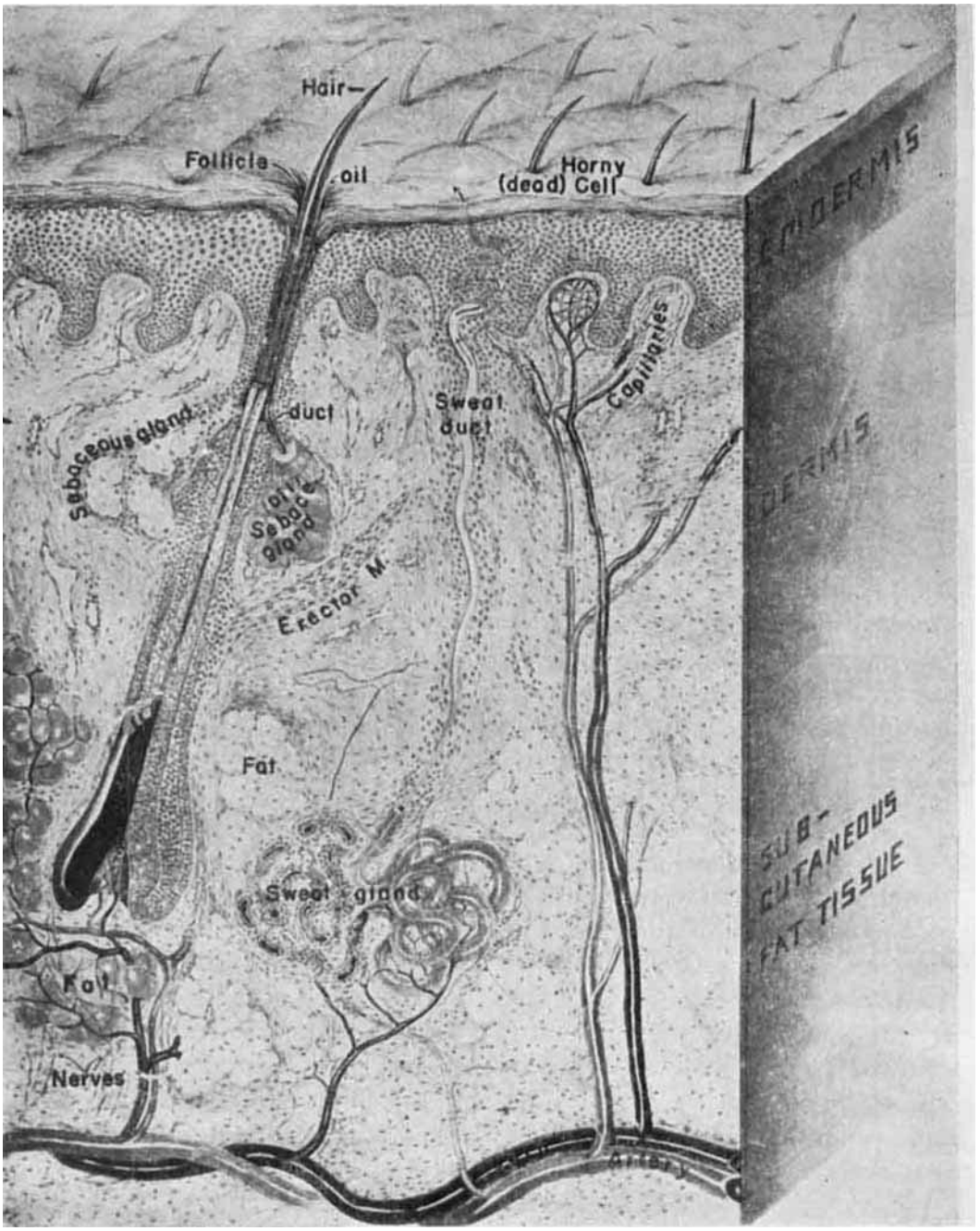


Fig. 1.—Anatomical zones encountered by a penetrating substance in transit from the skin surface to the blood vessels [from Griesemer, R. D., *J. Soc. Cosmetic Chemists*, **11**, 80(1960)].

lished autoradiograms of rabbit skin which had been exposed to the anticholinesterase agent, sarin, tagged with P^{32}. These indicate that penetration occurred through the transepidermal route. A similar route of penetration was described for hydrocortisone by Scott and Kalz (19).

It appears to be true that the ratio of the solubilities in water and in lipids, as originally advocated by Meyer and Overton, is actually important for the absorption of substances through the skin. Rothman (12) has shown that lipid-soluble substances penetrate the skin more readily than polar substances, and that substances soluble in both lipids and water penetrate most rapidly of all. Treherne (17) obtained quantitative data on the permeability of excised human skin to a series of radiotagged substances. He showed

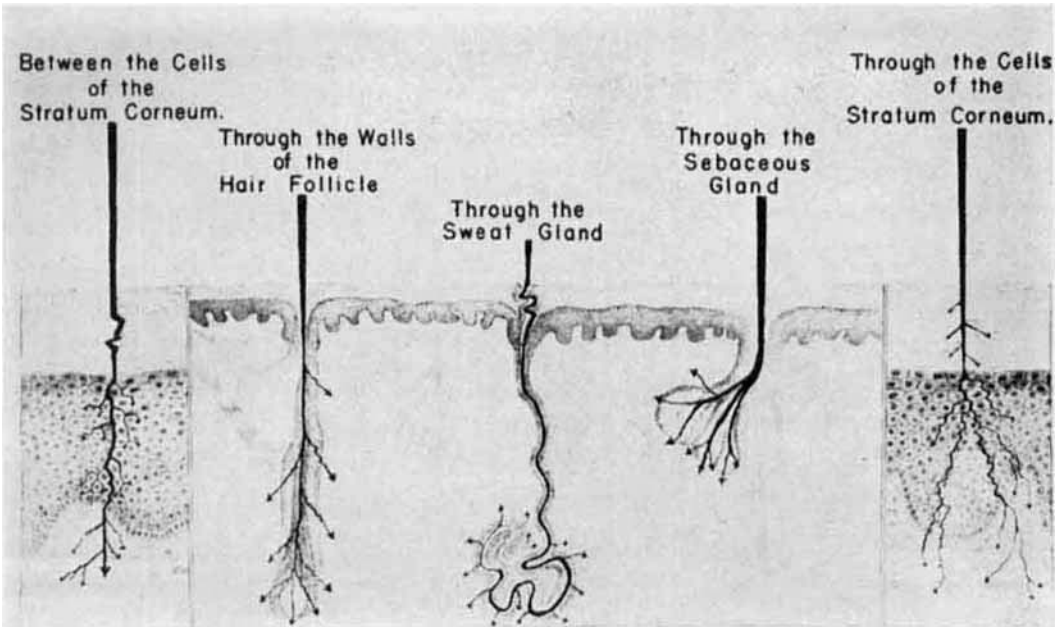


Fig. 2.—Possible avenues of penetration into and through the unbroken skin [from Griesemer, R. D., *J. Soc. Cosmetic Chemists*, 11, 81(1960)].

conclusively that the permeability of skin is directly proportional to the ether/water partition coefficients of these substances.

Absorption Through Appendages.—Hair follicles and sweat gland ducts open on the surface of the skin in the form of visible pores and these are believed to be avenues for the passage of medicaments through the skin. As has been previously stated, a majority of investigators believe that the hair follicles are the major avenue for percutaneous absorption.

Pilosebaceous Apparatus.—There are many studies which have shown the prominent role of the pilosebaceous apparatus in percutaneous absorption. This concept takes into consideration the solubility of the drug in sebum.

In the upper portion of the follicular canal, the hair shaft does not adhere to the follicular wall, and therefore a space is formed which is filled with horny scale and air. This interspace is continuous with the duct of the sebaceous gland. The sebum from this duct eventually empties into the interspace (20). Therefore, any medicament possessing a solubility in sebum may penetrate this space and reach inside of the sebaceous gland whose membrane is more permeable than the epidermal barrier. Similarly, the wall of the follicular sheath is less resistant to penetration than the surface epidermis. From the sebaceous glands and hair follicles, medicaments may penetrate downward into the corium and from there into the blood, thus bypassing the barrier. Medicaments may also move upward from the sebaceous glands into the epidermis but without penetrating the barrier layer. This pathway of absorption was demonstrated by MacKee, *et al.* (21), and by other workers (22, 23) who detected the presence of various tracers in the deeper parts of the hair follicles and in the sebaceous glands.

Sweat Ducts.—There is much controversy as to whether the sweat ducts serve as an avenue for percutaneous absorption. Rein (24) reported that he observed the staining of the pores of sweat ducts, like those of follicles, when he introduced dyes into the human skin by electrophoresis. Ichihashi (25) postulated that this "pore pattern" indicated absorption of the dye through the sweat ducts. Abramson, *et al.* (26–28), agreed with Ichihashi's conclusion and found the development of the sweat pore patterns dependent on the electric charge of the skin. On the other hand, Flesch (29) could not demonstrate penetration of dye into sweat ducts. Other studies (30–32) militate against any substantial penetration or percutaneous absorption through the palms and soles, which possess a predominance of eccrine sweat glands.

FACTORS INFLUENCING PERCUTANEOUS ABSORPTION

There are many factors which are thought to influence the rate and extent of percutaneous absorption. These will be discussed in this section.

Condition of the Skin.—The routes which a medicament may take in its passage through the intact skin have been discussed previously. If the skin is in an abnormal state, the limitations placed on the penetration of a medicament do not apply. If the barrier is destroyed by trauma, as in cuts, chapping, ruptured blisters, or in eczema, all substances pass freely into the dermis (9). Blank, *et al.* (18), have demonstrated by autoradiography that there is a striking increase in the penetration of sarin tagged with P^{32} following a superficial scratch extending just barely through the barrier. Similar data were obtained from trauma by adhesive tape stripping and puncture wounds.

Shelmire (33) pointed out that the vehicle of a topical preparation assumes more importance when the stratum corneum is intact and that differences in drug penetration attributable to the vehicle are more pronounced. On abraded or diseased skin there may be a large increase in both rate and extent of absorption of a drug in a vehicle (33).

It may be shown experimentally that the percutaneous absorption of materials coming in contact with the skin is significantly reduced in old age (34). This may be due in part to the atrophic changes of the pilosebaceous apparatus through which such substances are absorbed. It is a clinical fact that sensitization reactions to contactants are less commonly encountered in the older patient. In addition to lowered absorption, there may be a decreased capacity to form antibiotic conjugates in the skin or to form circulating antibodies.

Thermodynamic Properties of the Medicament.—The importance of the thermodynamic properties of a medicament as it relates to its percutaneous absorption has been discussed by Higuchi (35). These include the thermodynamic activity of the drug in the vehicle and in the skin barrier phase, and the diffusion coefficient of the drug in the vehicle and skin barrier phase.

Assuming that the vehicle containing the penetrating substance does not appreciably affect the skin, the following approximate relationship was set by Higuchi for an idealized system

$$\frac{dq}{dt} = (P.C.)\frac{(\text{concn. of drug})\,DA}{L} \qquad \text{(Eq. 1)}$$

where $(P.C.)$ is the effective distribution coefficient of the penetrating medicament between the vehicle and the barrier of the skin; (concn. of drug), the concentration of the medicament in the vehicle; D, the effective average diffusivity of the medicament in the barrier phase; A, the effective cross section area; and L, the effective thickness of the barrier phase. The main characteristics of the penetrating agent which determine its rate of entry through the skin, according to this equation, are its effective distribution coefficient and diffusivity in the barrier phase. The product of these two is referred to as the "permeability constant."

Actually, the important variable in the permeability constant of Higuchi is the effective distribution coefficient factor since the diffusivity of a substance of similar molecular weight and shape usually differs only slightly. The distribution coefficient, on the other hand, is an extremely sensitive function of molecular structure and size (35). Rothman (36) also emphasized the importance of the distribution coefficient of a penetrating substance between its vehicle and a membrane and between a membrane and its receptor solution on permeability. It depends chiefly on the force of molecular interaction between membrane and penetrating molecule. A substance can easily enter a membrane when the partition coefficient (membrane/vehicle) is high; it cannot easily leave the membrane when the partition coefficient (receptor solution/membrane) is low. In complex structures such as the skin, where the membrane may be nonpolar and the receptor-tissue fluids polar, a substance whose partition coefficient between polar and nonpolar solvents is nearly 1.0 will have the highest penetration rate. Thus, permeability is more rapid if there is some, but not too much, affinity between a penetrating molecule and a membrane. Under these conditions, the membrane will attract the penetrating molecule, but not so strongly that it fails to release it on the other side.

Higuchi (35) expressed Eq. 1 in another form in terms of the thermodynamic activity of the penetrating agent in its vehicle

$$\frac{dq}{dt} = \frac{A}{\gamma}\frac{DA}{L} \qquad \text{(Eq. 2)}$$

where a is the thermodynamic activity of the drug in its vehicle and γ is the effective activity coefficient of the agent in the skin barrier phase. Equation (2) shows the role of the activity of the drug in its vehicle while the properties of the base itself seem to play no part. For such systems the rate of percutaneous absorption measured for different ointment bases would be approximately constant provided the thermodynamic activity of the drug in the vehicles was maintained constant. Thus, all ointments containing finely ground suspensions of the drug

(thermodynamic activity equal to that of the solid drug) will produce the same rate of penetration. This again presupposes that the rate-determining step is essentially in the passage of the barrier phase. Since activities are more important than any absolute concentration, it is obvious that, for a given concentration of a penetrating substance, vehicles which have lower affinity (poorer solvent power) will normally produce faster penetration (35). For additional discussions on the absorption of medicaments from suspension and solution, readers are referred to works by Higuchi (35) and Wagner (37).

Effect of Moisture.—There are two opposite views on the influence of moisture on percutaneous absorption (38). Based on the concept that the skin is relatively impermeable to water, one school assumes that moisture on the outside has little to do with promoting percutaneous absorption. On the other hand, since it has been a long-standing clinical experience that watertight covering of the skin surface does influence percutaneous absorption, another school believes that moisture is a most important factor in promoting the passage of medicaments through the skin.

Renshaw (39) and Cullumbine (40) reported that much more severe lesions develop on human skin if mustard gas and nitrogen mustard gases are applied to wetted skin than to dry skin. Their work was interpreted by some to indicate that moisture plays an important role in promoting skin penetration. However, it is known that, in contact with water, mustards transform to various highly soluble, reactive, and toxic intermediary products before the inactive end products of the water-mustard reactions are formed (41, 42), and these may be responsible for the production of the severe lesions.

Laug, *et al.* (43), demonstrated that increased moisture promotes transfollicular absorption. They found that the absorption of mercury is increased fourfold by covering the site of inunction. It was assumed that the cover acts by interfering with the normal loss of moisture. Shelley and Melton (44) found that the application of histamine solution to the skin produced a prolonged effect when the site was covered, a result also probably due to the inhibition of water evaporation. Leslie-Roberts (45) concluded that moisture causes maceration of the horny layer and, therefore, provides a condition promoting the retention of substances in contact with the skin.

Recent work has more definitely established the importance of moisture in promoting percutaneous absorption. Higuchi (35) has stated that the transfer properties of several layers of the skin are probably strongly influenced by the presence of water since it is well sorbed by protein and protein degradation products contained in the outer skin. He demonstrated this type of behavior using artificial membranes. He has shown experimentally that, at very low humidity, the permeability of glyceryl monostearate is relatively insensitive to relative humidity, whereas near 100%, the rate of penetration is actually dependent on water activity. This is attributed to imbibition of water by the barrier phase exposed to saturated water vapor and consequent changes both in the diffusion coefficient and activity coefficient. Wurster and Kramer (46), investigating the absorption of three salicylate esters, demonstrated that a large increase in its absorption rate was produced by increased moisture conditioning. They assumed that percutaneous absorption involves a diffusional process, i.e., the spontaneous movement of a substance from an area of high concentration to an area of low concentration in the tissue fluids. They concluded that the magnitude of the effect of moisture on percutaneous diffusional rates has been shown to be proportional to the oil/water distribution coefficient and the water solubility of the test substance. The greatest increase of the penetration rate by moisture was found in the penetrant with the smallest oil/water distribution coefficient.

Shelmire (33) stated that hydration of the stratum corneum is one of the most important factors in the penetration of skin by a medicament. The hydration of the stratum corneum appears to increase the rate of passage of all substances which penetrate the skin. He (33) suggested that the mechanism was to increase the size of the pores. There will not only be a physical alteration of the tissue due to hydration but also at high water activities there will be changes in both the diffusion coefficient and activity coefficients of the penetrating agent (35). Shelmire (33) believes that the nature of the vehicle upon application and, when the water in it has evaporated, may markedly alter the activity of water in the stratum corneum.

Greases and oils are the most occlusive vehicles and induce the greatest hydration through sweat accumulation at the skin-vehicle interface (33). Emulsions of the water-in-oil type are somewhat less occlusive than greases (33). Oil-in-water emulsions tend to invert as the outer aqueous phase evaporates, and the final state may be considered to be a continuous oil film, containing other dissolved or suspended substances (33).

Humectants in such emulsions tend to decrease the extent of hydration of the stratum corneum by interference with the formation of a continuous oil film on the skin surface (33). Water-soluble vehicles produce the least change in hydration of the stratum corneum (33). Hydrophilic powders will decrease the extent of hydration by increasing surface area and, hence, increasing the rate of evaporation of water (33). Hydrophobic powders probably cannot retain moisture because they form a discontinuous film (33). All powders probably tend to interfere with the continuity of oil films and, in this manner, probably decrease the occlusive effect of any vehicle in which they are used (33). It should also be remembered that if an ointment is covered with a bandage there will be a tendency to hold perspiration and increased hydration; if left open to the air the perspiration can evaporate and hydration may occur (33). The thickness of the applied film of topical preparation will also directly affect hydration of the stratum corneum (33).

Effect of Vehicles.—There have been many studies on the role that a topical vehicle plays in the percutaneous absorption of drugs but until recently this subject has been somewhat in a state of confusion. This has been due in part to deficiencies in many of the methods used for measuring the extent of percutaneous absorption and to a lack of consideration of the thermodynamics involved in the interpretation of results. The thoughtful discussions by Higuchi (35), Shelmire (33), and Wagner (37) have clarified many aspects of this subject. They have thrown new light on the results obtained by workers in the field and indeed on the entire subject of percutaneous absorption.

It has been thought by a majority of workers in the field that the function of the vehicle in percutaneous absorption is to facilitate contact between the medicament and the absorbing cells. Absorption is best from vehicles which spread easily over the skin surface, readily mix with the sebum, and so bring the medicament into contact with the absorption cells. It was believed at one time that the primary factor influencing penetration through the skin was the vehicle itself. Thus the subdivision of ointment bases into epidermic, endodermic, and diadermic types became popular (47). It gradually became apparent that the thermodynamic activity and the diffusion coefficient of the medicament in the vehicle and skin barrier phase (35), the thermodynamic activity of moisture in the vehicle and skin barrier phase (35), and the degree of hydration of the skin (33) were of greater significance in influencing percutaneous absorption. It is believed that the vehicle itself is not capable of promoting the absorption of nonabsorbable drugs dispersed in it, but rather may modify the absorption properties of absorbable drugs (48).

In this section, some of the studies on the effect of vehicles on the percutaneous absorption of medicaments will be reviewed.

It has long been known that absorption of medicaments is better from animal and vegetable oils than from mineral oils because they readily penetrate the skin (49–51). Organic solvents like ether, chloroform, benzene, and acetone penetrate the skin with ease (52) and enhance the percutaneous absorption of a drug (53) to such an extent that toxic effects can occur. On the other hand, it has been demonstrated that defatting the skin with ether decreased the absorption rate of methyl salicylate (46). Higuchi (35) stated that the application of many solvents other than water appears to cause marked alteration in the resistance of the skin barrier toward penetration and that this phenomenon is possibly caused by marked changes produced by such solvents in the activity coefficient and diffusion constant of the penetrating agent in the skin barrier.

Strakosch (54) concluded from his histological studies that emulsion type bases do not have any effect on the degree of percutaneous absorption of salicylic acid. In contrast, results obtained from patch tests (1, 55), and analyses of urine (56, 57), and blood samples (58) obtained after topical application of ointment vehicles containing salicylic acid indicated differences in absorption from various emulsion-type bases. Similar contradictory results may be noted in percutaneous absorption studies of ointment vehicles containing sulfonamides (59–63).

Nogami, *et al.* (64), stated that the influence of the ointment base on the percutaneous absorption of salicylic acid was not great but that its absorption from hydrophilic ointment was less than from soft paraffin, simple ointment, and absorption ointment.

Bourget (65) and Kimura (66) made a quantitative study of the effect of different fatty bases on the percutaneous absorption of salicylic acid as judged by excretion of the drug. It was found that salicylic acid was rapidly absorbed and the amount depended on the vehicle; the ones giving the greatest absorption being lard and lanolin; the least effective, petrolatum.

Many workers have reported that the type of vehicle is of importance in the absorption of

mercury (22, 43, 67–69). However, Sollmann, *et al.* (70), have contradicted these reports.

Using radioactive iodine (71), radioactive sodium iodide (72), and potassium iodide (73), several investigators concluded that these substances appear to be more efficiently absorbed from wool fat than from lard. A difference in the type of emulsion vehicle had practically no effect on the absorption of the iodide.

Oil-in-water emulsions as well as water-soluble vehicles deserve special mention because of the claims that they would promote percutaneous absorption of drugs due to either the presence of surfactants or their water-miscible properties (43, 74). Eisner (75) demonstrated that a continuous oil phase would tend to reduce percutaneous absorption, a fact contradicted quite recently by the work of Gemmell and Morrison (60). There is no doubt, however, that a continuous water phase would be miscible with the secretions of the denuded and oozing surfaces of the abraded skin, and thereby promote the release of the medication. This was demonstrated by Zeutlin and Fox (76) who concluded that the release from water-soluble and oil-in-water type vehicles was far superior to the release from oleaginous and water-in-oil type vehicles. Strakosch and Clark (61) and Laug, *et al.* (43), supported with their experiments the theory of superiority of emulsion-type vehicles to oleaginous vehicles; they could not, however, show a significant difference in absorption for oil-in-water or water-in-oil emulsion vehicles while working with mercury (43) and sulfonamides (61). In contrast to this, a superiority of a water-in-oil vehicle to an oil-in-water vehicle was demonstrated by Gemmell and Morrison (60) who studied the absorption of sulfonamides.

It should be remembered that a great majority of percutaneous absorption studies have been carried out on animals. There may be a considerable difference in the rate and extent of absorption of a given drug in various species of animals and in man. The use of many types of test subjects is a further reason why results obtained from percutaneous absorption studies are somewhat confusing.

Effect of Surfactants.—Surfactants offer possibilities of improving topical vehicles and promoting a more thorough diffusion of the medicament from the vehicle, thus influencing therapeutic performance (77–78). Sweet (79) suggested that the mode of action for surfactants was that they are capable of emulsifying the sebum and, therefore, increase percutaneous absorption in this manner.

Duemling (74) reported that deeper and more rapid penetration of ammoniated mercury is obtained by the addition of a wetting agent to a paraffin ointment base. Meyers, *et al.* (80), investigated the absorption of potassium iodide and of phenolphthalein from various ointment bases through the intact skin of albino rats. They found no promoting action to be produced by the wetting agent, sodium tetradecyl sulfate.[1] MacKee (30) demonstrated penetration through intact skin of ferric chloride, bismuth (sobisminol), and sulfonamide from a series of topical vehicles called "penetrasols." These bases are composed of propylene glycol, antipyrine, xylene, and sulfonated wetting agents of the fatty acid esters of sulfosuccinic acid type. Shelley and Melton (32) confirmed the fact that these vehicles promote the percutaneous absorption of water-soluble drugs. In addition, they demonstrated that absorption occurred only from areas containing hair follicles. By analyzing the liver and kidneys of rats for mercury, Laug, *et al.* (43), compared the absorption of mercury from various ointment bases, some of which contained a surfactant, and found a significant increase in percutaneous absorption from the vehicles containing these agents. Duemling (74) demonstrated rapid and complete penetration of mercury from vehicles containing sodium lauryl sulfate. The incorporation of cholesterol in liquid petrolatum seemed to increase the penetrating powers of the vehicles (81), but the application of cholesterol to the skin appeared to reinforce the resistance of the fatty substances normally present in the skin and, therefore, reduced the percutaneous absorption of water-soluble drugs (82).

Stolar, Rossi, and Barr (83) detected a marked reduction in the percutaneous absorption of salicylic acid in hydrophilic ointments as the concentration of surfactants having polyoxyethylene groups was increased. They concluded that this is due to the complexing of the salicylic acid by the polyoxyethylene groups, i.e., salicylic acid has a low thermodynamic activity in these vehicles.

Stark, *et al.* (84), studied the influence of the nature of the surfactant in ointment bases on the release of various substances using an *in vitro* technique utilizing a membrane. Radioactive mercuric (Hg^{203}) and iodide (I^{131}) ions were incorporated into hydrophilic ointments emulsified with various concentrations of anionic, cationic, and nonionic surfactants. It was found

[1] Marketed as Tergitol 4 by Union Carbide Chemicals Co., 270 Park Ave., New York 17, N. Y.

that the ionic or nonionic nature of the surfactants substantially influenced the release. The nonionic-type surfactants promoted the release of both the iodide and mercuric ions to a greater extent than did the anionic or cationic types. Vinson and Choman (85) investigated the relative effects of surfactants on the cutaneous penetration of nickel sulfate through the skin of guinea pigs. They found that whereas the nickel sulfate did not penetrate when applied alone, sodium lauryl sulfate and sodium dodecyl benzyl sulfate did provoke its penetration.

Effect of Concentration of Medicament.—Strakosch and Clark (61) reported that increasing the concentration of sulfonamide from 1 to 10% in the vehicle caused no increase in their absorption when applied to the skin. It was similarly demonstrated by Laug, *et al.* (43), that the concentration of mercury in different ointment vehicles has very little effect on the degree of percutaneous absorption.

Higuchi (35) emphasized the role of concentration of a drug on its rate of absorption from a suspension-type ointment. He presented the following equation

$$\frac{dQ}{dt} = \sqrt{\frac{ADC_s}{2t}}$$

where A is the concentration of the drug expressed in units/cm.^{3}; C_s, the solubility of the drug as units/cm.^{3} in the external phase of the ointment; D, the diffusion constant of the drug molecule in the external phase; and dQ/dt, the rate of absorption. He concluded that the rate of release of drugs from such preparations can be regulated by controlling A, D, and C_s.

Effect of pH.—The rates of absorption of acidic and basic drugs are strongly influenced by the effective pH of the vehicle (35). The activity coefficient of the molecular form of such drugs is a rapidly changing function of pH for pH values greater than pKa for acidic compounds and less than pKw-pKb for alkaloidal drugs. Thus, as Higuchi states (35), the rate of absorption of histamine would be 10 times greater from a base buffered at pH 7.5 than from a base at 5.5, assuming that only the nonionized molecule is involved in the absorption process.

Bhatia and Barber (86) evaluated the effect of pH on the local anesthetic activity of benzocaine by measuring the pain threshold. They concluded that the maximum local anesthetic activity appeared to occur between the pH of 6 and 7, and decreased when the pH was varied. This was substantiated by Roques, *et al.* (87). Other workers (66, 88) concluded that systemic alkalosis increased the percutaneous absorption and excretion rates of various drugs while systemic acidosis decreased the rate of percutaneous absorption and excretion.

Miscellaneous Factors.—Other factors which influence the rate and extent of percutaneous absorption of medicaments include: the route of absorption (33, 35); the area to which the drug is applied (33, 35); the effective thickness of the skin barrier phase (35); and the individual contact time and frequency of reapplication (33).

It is well known that the elevation of skin temperature enhances percutaneous absorption. This may be due to a lowering of the viscosity of the sebum which would facilitate its mixing with the inuncted preparation. Furthermore, a rise in skin temperature also increases the cutaneous circulation through vasomotor dilation of the skin vessels. Blank, *et al.* (89), reported that the rate of penetration of sarin increases approximately twofold for a 10° rise in temperature. And finally, Higuchi (35) pointed out that the diffusion coefficient of a medicament, D, is inversely proportional to the viscosity of the vehicle.

METHODS OF MEASURING PERCUTANEOUS ABSORPTION

The results obtained by the various investigators who have studied the percutaneous absorption of drugs have been, for the most part, quite contradictory, probably due to inadequate and in many cases uncontrolled measuring procedures. There have been several excellent papers published in recent years in which the methods of measuring percutaneous absorption have been reviewed (3, 90–92).

Some authors classify the methods employed in measuring percutaneous absorption into *in vitro* and *in vivo* procedures. *In vitro* methods are of limited value but they are a means of assessing the ability of a vehicle to liberate the medicament under the conditions of the test. However, these methods can be, for the most part, dismissed as being quite useless in providing reliable information on percutaneous absorption. On the other hand, *in vivo* methods give a more accurate picture of percutaneous absorption, although the results obtained from such methods are often confusing.

Blank (3) has proposed a new classification of the various methods which have been used in studying percutaneous absorption. His classification will be used in this review.

Penetration into Models.—All measuring methods using models are of the *in vitro* type. They are of limited value but they are a means of assessing the ability of a vehicle or base to liberate medicament under the conditions of the test. Those reported are of a comparative nature and most are empirical, which prevents results from being compared with those from other techniques. Lockie and Sprowls (93) hold that the study of ointment bases by *in vitro* methods is essentially a study of diffusion rates.

The most common model which has been used to simulate skin is agar gel. It has been commonly used in studying the release of antibacterial agents from ointment bases. It has been too often assumed that those ointment bases which best release antibacterial agents to an agar gel will similarly release these substances to the skin when the ointments are placed on the skin. This is not so since the continuous phase in agar gel is water, and the skin is a much more complex chemical and physical structure.

Membranes composed of cellulose film (94) and sheep's bladder (95) have also been employed as skin models in *in vitro* procedures. While the assumption is made that the process of penetration in the skin is similar to the quantitative diffusion through a membrane, this does not make any allowance for differences in physicochemical properties of "dead membranes" and living tissue, the latter presenting a much more complex system, both physically and chemically.

Histological Studies.—A variety of procedures under this heading has been adopted to evaluate drug release from vehicles. Strakosch (96) has studied the penetration of salicylic acid, sulfur, resorcinol, and other substances by examining histological sections for evidence of keratolysis. No effort was made to measure such penetration quantitatively. Duemling (74) also utilized histological studies to measure the absorption of medicaments. Goldzieher, *et al.* (97), used histologic sections in their study of the penetration of estrogen. Histological studies made by Sobel, *et al.* (98), indicated that vitamin A is absorbed and utilized by the skin.

Use of Tracers.—Dyes and fluorescent materials have been utilized for a long time in percutaneous absorption studies. In recent years use has been made of radioactive substances as tracers in such studies.

Dyes.—One of the early methods used for studying penetration of substances into skin employed an oil-soluble dye to color the material being studied (99–102). Histological sections of the skin were then examined for the presence of dye. This method is subject to much criticism as one cannot be certain that the dye remains with the substance whose penetration is being studied.

Fluorescent Compounds.—The use of fluorescent compounds (100, 103) in penetration studies is open to the same criticism as those in which a dye is used as a tracer. One cannot be sure that the fluorescent material remains with the substance whose penetration is being studied. This objection is not valid if the penetrating substance itself is fluorescent.

Radioactive Tracers.—The use of radioisotopes has become increasingly popular for studying percutaneous absorption (104). In such procedures an attempt is often made to synthesize the substance being studied so that it includes a radioisotope. Sometimes the molecular structure of the penetrating substance is modified somewhat by incorporation of the radioactive element, as for instance in the iodination of an unsaturated fat with I^{131} (100). Most of the percutaneous absorption studies involving the use of radioactive tracers are carried out by one of the following techniques: (*a*) direct counting at the surface; (*b*) autoradiographic technique; and (*c*) measurement of the radioisotope within the body.

The direct counting technique is performed by counting the amount of radioactivity on a given area of the surface of the skin by using an end window Geiger tube if the substance under study is an alpha- or beta-emitter of sufficient intensity. One such investigation (105) involved the application of 0.005 ml. of an alcoholic solution of thorium X, with an activity of approximately 2,000 disintegrations per minute, to four areas on the forearm. The activity on each of these areas was counted with an end window tube immediately following the application of the solution and 24 hours after application. The loss in activity represented the amount of thorium X which was absorbed. Ainsworth (92) described a method for studying percutaneous absorption of a radioactive compound by following its disappearance from the skin surface.

Autoradiographic technique, first developed by Axelrod and Hamilton (106), is a very useful procedure for demonstrating the penetration and localization of radioactive materials providing they emit alpha and beta rays. In order to prepare an autoradiogram, a biopsy must be made and tissue sections cut for transfer to nuclear track emulsions. The particular tech-

nique which is used will be determined by the type of radiation, the half-life, and the solubility of the radioactive isotope being studied. The penetration of thorium X (7, 107) and sarin (18) have been studied by this method and it was found that the penetration of the first is by way of the appendages and the penetration of the second is primarily transepidermal. Choman (108) has reported on an improved autoradiographic technique in which he measured the percutaneous absorption of sodium lauryl sulfate (S^{35}) and nickel chloride (Ni^{63}). An investigation of the pathways of absorption of hydrocortisone (C^{14}) free alcohol was carried out by the technique of autoradiography by Scott and Kalz (19).

In working with autoradiography one should be familiar with the following pitfalls. When a radioactive element that emits high energy radiation is being used, there will be a considerable scatter on the autoradiogram. Secondly, reducing substances in the tissues may react chemically with the emulsion and produce a shadow even when no radioactivity is present. Third, a shadow may be produced in the autoradiogram even when no radioisotope is present if the tissue and photosensitive emulsion are on separate slides which, during exposure, are clamped tightly together.

Percutaneous absorption has been studied by measuring the concentration of a radioactive substance which has been absorbed in the blood, urine, expired air, thyroid gland, and hair.

Hadgraft, *et al.* (109), have studied percutaneous absorption in rats by measuring the concentration of diiodofluorescein (I^{131}) in the blood. Hupf, Chase, and Barr (110) studied the effect of various ointment bases on the percutaneous absorption of salicylic (carboxyl C^{14}) acid through intact rabbit skin by measuring blood concentrations of the radioactive substance.

Goldzieher and Baker (111) investigated the absorption of estradiol 17-β (H^3) and progesterone (C^{14}) through the skin of guinea pigs by measuring the concentrations of the radioactive substances in urine. Hellman, *et al.* (112), Malkinson and Ferguson (113), Livingood (114), and Malkinson, *et al.* (115), studied the percutaneous absorption of topical corticosteroids (C^{14}) through human skin by measuring the concentrations of the radioactive substances in the urine.

Lange and Evans (116) applied an ointment containing radon to patients and the amount of radon in the expired air was taken as a measure of the amount passing into the bloodstream.

Cyr, *et al.* (117), and Skauen, *et al.* (72), studied the effect of ointment bases on the absorption of sodium iodide (I^{131}) through the skin of rats by measuring the concentrations of the radioactive substance in thyroid glands which were removed from the animals.

Edwards (118) has studied the absorption of topically applied amino acids in guinea pigs using methionine (S^{35}) by measuring the concentration of radioactive material in newly grown hair.

Measurement of Physiological Reactions.—The percutaneous absorption of certain drugs in ointment form has been studied making use of their pharmacological effects or end points. Those which have been used include a rise in blood pressure (119), measurement of vasodilation (120), red tear response (121), production of anesthesia (122), reduction in pain threshold (123–125), production of convulsions (126), production of urticaria (127, 128), vasodilation (129), and death (122, 129).

The effect of passage of hormones through the skin has been the subject of wide research; a few of the many references will be quoted. Different criteria have been used for the measurement of percutaneous absorption of hormones. These consisted of the growth of testes (130), development of a scrotal hernia (131), mammary growth (132, 133), the increase in weight of the seminal vesicles and the prostate gland (134, 135), increase in growth rates of feathers and comb of a capon (135), and the ejaculation reflex as well as the weight of the ejaculate (133, 136).

Among the methods used for studying the percutaneous absorption of vitamins may be mentioned those based on the dark adaption test for vitamin A (137), the thickening of skin in rats by vitamin A (138, 139), weight gain and increase in growth caused by vitamin A in vitamin A-deficient rats (98, 140, 141), and curative effects in man for vitamin C (142), vitamin D (143), and panthenol.

Analysis of Tissues: Skin, Blood, Urine, etc.—Probably the most common method of studying percutaneous absorption *in vivo* is by analysis of the circulating blood (58, 60, 110, 144), urine (145–150), and feces (151) at different intervals following application of the material to the cutaneous surface. One of the difficulties encountered in attempting to estimate quantitatively the amount of material which has penetrated the skin by analyzing the blood, urine, or feces is the fact that the rate at which material leaves the blood stream and is excreted or stored in the tissues is seldom known.

In those cases where drugs appear to have a greater affinity for a particular organ, the organ may be excised and analyzed for drug content. This principle has been used by some workers for the determination of the percutaneous absorption of mercury (43, 152–154), iodides, and iodine (117, 155, 156).

Loss of Penetrating Substance from the Surface.—If a known amount of a nonvolatile substance which has been allowed to remain on the cutaneous surface for a definite period of time is quantitatively removed from the surface and analyzed, the difference between the amount applied and the amount removed may be thought to have penetrated the skin (157). Blank (3) discusses several pitfalls in this method. Quantitative removal is difficult. Some materials combine chemically with the constitutents of the cornified epithelium and then cannot be easily separated from it although they have not penetrated into skin any further than the stratum corneum. The amount of most substances which penetrates is often so small that errors in analysis may be of the same order of magnitude as the amount absorbed and, therefore, one can never be sure that the difference between "before" and "after" determinations truly represents the amount of material which has penetrated. Now that radioactive substances can be prepared it is possible to determine loss from the surface simply by determining the decrease in radioactivity. This subject has been discussed previously.

PERCUTANEOUS ABSORPTION OF MEDICAMENTS

The consensus of the majority of investigators who have studied the percutaneous absorption of medicaments appears to be that the greater the solubility of the drug in oil than in water, the greater its percutaneous absorption. However, it should be emphasized that some degree of solubility of a medicament in both oil and water is thought to be essential for it to be absorbed percutaneously. This phenomenon is not specific to percutaneous absorption, but holds true to absorption and action of drugs in general (158).

The following drugs, all more oil-soluble than water-soluble, have been reported to be absorbed percutaneously: benzocaine (86), iodine (117, 145, 155, 156), phenolsulfonphthalein (159), sulfathiazole (160), sulfanilamide (60, 61), sulfadiazine (161), ammoniated mercury (67), mercuric oxide (43), salicylic acid (45, 54, 58, 83, 153, 162–164), methyl salicylate (163), glycol salicylate (163), diiodofluorescein (109), pyribenzamine (165), morphine (166), strychnine (126, 166), pilocarpine (166), aconitine (126, 166), acetylcholine oleate (167), eserine (121), nicotine (129, 166), stearic acid (100), mustard gas (40, 101), phenol (168–172), and sarin (18, 89).

Although it is thought by many that predominantly water-soluble drugs are not absorbed percutaneously, there are many references in the literature which report that such absorption does take place. Among the water-soluble drugs which have been proved to be absorbed percutaneously are: tritium oxide (173, 174), deuterium oxide (175), sodium chloride (146, 176), thiamine hydrochloride (12), vitamin C (12), mercury bichloride (177), morphine sulfate (166), strychnine sulfate (12), pilocarpine nitrate (32), acetylcholine chloride (32), epinephrine hydrochloride (32), histamine phosphate (32), sodium iodide (71, 72), potassium iodide (71, 178, 179), pyribenzamine hydrochloride (180), penicillin G potassium (181), chlorcyclizine hydrochloride (182), and sodium salicylate (6, 183–186).

Much work has been carried out in recent years on the percutaneous absorption of vitamins, corticosteroids, and sex hormones, and these will be reviewed.

Vitamins.—The percutaneous absorption of vitamins has been reviewed recently by Rubin (187). In brief, vitamin A has been shown to be absorbed percutaneously in animals (53, 140, 141, 189–191) and in man (137, 191). Vitamin D has been shown to be absorbed percutaneously in animals and humans (192, 193); vitamin K in infants (194); and vitamin E in the rat (195). All four of these vitamins are oil-soluble and this property undoubtedly is largely responsible for its absorption.

Of the water-soluble vitamins, panthenol has the largest literature concerning percutaneous absorption. Absorption has been demonstrated in animals (196, 198) and in man (198, 199). The percutaneous absorption of vitamin B_6 (200, 201), thiamine hydrochloride (200, 202), riboflavin (200), calcium pantothenate (200), and vitamin C (203) has also been reported.

Sex Hormones.—The effectiveness of estrogens and progesterones in dermatology and in cosmetics is based on their percutaneous absorption. The rate of absorption of these substances is primarily determined by the vehicle (204).

The most widely used hormones in cosmetics are the estrogens. These substances have produced demonstrable results in the treatment of acne in both sexes. Topical estrogens were also

observed to have a restorative effect on distorted elastic fibers common in aging skin. Among others, Masters (205) and Goldzieher and Baker (206) have demonstrated the percutaneous absorption of estrogens.

Progesterone has been reported to penetrate the intact skin rapidly and with great ease (206, 207). The absorption of progesterone appears to be more rapid than with estradiol (206). Both testosterone and its propionate ester are absorbed through the skin, the free alcohol being more effectively absorbed than the ester (208).

Corticosteroids.—Much interest has been shown in recent years on the percutaneous absorption of the corticosteroids. This subject will be reviewed here.

Earliest studies to demonstrate the percutaneous absorption of hydrocortisone were based either on the physiological effects it produces or an increase in its metabolites in plasma or urine. Smith (209) found no significant change in the circulating eosinophile count following the application of 150 mg. of hydrocortisone acetate to normal or clinically inflamed skin of patients. In another investigation (210), this author failed to note any changes in the urinary excretion of 17-ketosteroids and 17-hydroxycorticosteroids in similar-type subjects. Witten, *et al.* (211), studied the blood and urinary levels of 17,21-dihydroxy-20-ketosteroids in humans for 3 days in response to external application of hydrocortisone acetate-containing ointments without finding significant changes. In another study, Fitzpatrick, *et al.* (212), could not demonstrate the absorption of hydrocortisone acetate in a lotion vehicle following its application to the skin of patients. As a result of these studies, it became apparent that for humans there was little danger from the absorption and consequent systemic effects of topical hydrocortisone in concentrations of 1 to 2.5%.

The first direct demonstration of the percutaneous absorption of hydrocortisone was carried out by Malkinson and Ferguson (113) who demonstrated the radioactive 17-ketosteroid fraction in the urine following the topical application of hydrocortisone (4-C^{14}) to normal human skin in the form of an ointment. Livingood, *et al.* (114), confirmed the absorption of hydrocortisone (4-C^{14}) in humans. Liddle (213) and Paulsen and Rerup (214) also demonstrated the percutaneous absorption of hydrocortisone in humans.

Although it had been shown that hydrocortisone penetrates the superficial barrier (19), Malkinson (215) found that removal of the barrier layer tremendously enhances the penetration of this hormone.

Investigations of the type previously described for hydrocortisone have been carried out with cortisone. Danto and Maddin (216) did not observe any change in the number of circulating eosinophiles in humans after the topical application of cortisone acetate. Malkinson and Ferguson (113) detected the radioactive 17-ketosteroid fraction in the urine following the topical application of cortisone (4-C^{14}) to normal human skin in ointment form. No increase in the penetration of cortisone (4-C^{14}) acetate occurred upon application to stripped sites (215).

Fitzpatrick, *et al.* (212), reported five cases of increased weight and ankle edema in patients treated with 0.2% fluorohydrocortisone acetate lotion. Livingood, *et al.* (217), found edema, weight gain, and/or diminished urinary excretion of sodium in a group of 11 patients receiving topical fluorohydrocortisone. Although it is apparent that fluorohydrocortisone is absorbed percutaneously, as yet there is no decisive evidence of its penetration through normal skin.

There has been very little carried out on percutaneous absorption studies of other corticosteroids.

SYSTEMIC TOXICITY RESULTING FROM PERCUTANEOUS ABSORPTION

Investigations for potential systemic toxicity resulting from percutaneous absorption must be considered as simply one aspect of the overall evaluation of safety which is carried out to determine whether products are safe for patient use (218). The systemic toxicity resulting from the absorption of medicaments applied to the skin is influenced by two independent properties: (*a*) the ability of the medicament to penetrate the skin and (*b*) the inherent toxicity of the medicament toward the patient. Systemic toxicity can occur only when the medicament in question is both absorbed and toxic. However, dangerous toxic symptoms may result on application of potent medicaments to large areas of broken skin. Also of importance are the concentration of medicament and the area of broken skin to which it is applied.

Systemic toxic reactions are important from the standpoint of both acute and chronic effects. Acute toxicity is easier to measure and the results are easier to interpret. On the other hand, in studying the chronic effect of a medicament, it is important to relate the induced changes to the dosage and the frequency of application to the subject. On this basis, the possibility of a similar human exposure may be drawn.

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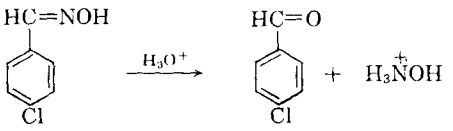
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Facile Acid Hydrolysis of *p*-Chlorobenzaldoxime and Its Oral Inefficacy

By EDWARD R. GARRETT†

The kinetics of acid hydrolysis of *p*-chlorobenzaldoxime have been studied. A polarographic assay for reactant and product has been devised. The oxime readily hydrolyzes in acid solution to form an equilibrium mixture with the products. It is expected that the half-life of this oxime would be less than 20 minutes in the human stomach and that the oral inefficacy may be ascribed to this mode of degradation.

OXIMES have been shown to have interesting pharmacological properties (1). In recent animal work at our laboratories, a typical oxime, *p*-chlorobenzaldoxime (PCBO), was inactive as a muscle relaxant (2, 3) when administered orally but active when administered parenterally. The facile hydrolysis of the oximes to *p*-chlorobenzaldehyde (PCB) and hydroxylamine at the pH of the stomach may be a possible explanation.



This paper considers the appropriate assay for such kinetics of hydrolysis and the validity of this possible explanation of oral inefficacy.

EXPERIMENTAL

Choice of Assay Method.—The change of the ultraviolet absorption spectra of *p*-chlorobenzaldoxime in hydrochloric acid solution was extremely small and surprisingly fast. The asymptotic value of the spectra with time was too close to the initial spectra. This made it necessary to investigate a more sensitive method of following the possible transformation of the *p*-chlorobenzaldoxime in hydrochloric acid solution to the *p*-chlorobenzaldehyde and the hydroxylamine hydrochloride.

The method chosen was polarography with the Leeds and Northrup dropping mercury electrode Electrochemograph.

Typical polarographic waves for the reactant and the possible products are given for 10^{-3} *M* solutions in 10% ethanol, 0.100 *M* hydrochloric acid at 25° in Fig. 1. Curve A is the diffusion current in microamperes, i_D, *vs.* volts for *p*-chlorobenzaldoxime, $E_{1/2} = -0.64$; i_D/M at -0.75 v. is $(10.98 - 0.90) \div 10^{-3} = 10.08 \times 10^3$ μa./mole/L. where the blank has 0.90 μa. at -0.75 v. Curve B for *p*-chlorobenzaldehyde shows an $E_{1/2} = -0.855$ v., i_D/M at -1.00 v. is $(3.50 - 1.08) \div 10^{-3} = 2.42 \times 10^3$ μa./mole/L. where the blank has 1.08 μa. at -1.00 v. Curve C for the hydroxylamine at 10^{-3} *M* indicates that this product of the hydrolysis will not significantly interfere with the polarographic assay of the *p*-chlorobenzaldoxime and the *p*-chlorobenzaldehyde. Table I gives the $E_{1/2}$ values after accounting for the i_D of the blank solutions for these two compounds in various hydrochloric acid solutions.

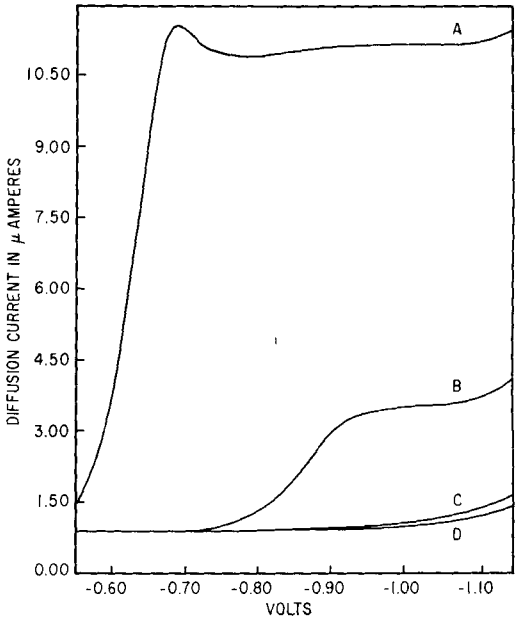


Fig. 1.—Polarographic waves at $10^{-3}M$ in 0.100 *M* hydrochloric acid and 10% ethanol-water at 25°. Curve A, *p*-chlorobenzaldoxime; curve B, *p*-chlorobenzaldehyde; curve C, hydroxylamine hydrochloride; and curve D, blank solution.

Figure 2 is the calibration curve for the i_D at -0.75 v. for *p*-chlorobenzaldoxime in 0.100 *M* hydrochloric acid, 10% ethanol, 25°, as a function of the oxime concentration. The i_D values are corrected for the i_D of the blank under these conditions. The slope or the specific i_D is 0.0633 μa./mcg./ml.

Figure 3 is a typical series of polarograms with time of the reacting solution, 10^{-3} *M* in *p*-chloro-

Received September 7, 1961, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication November 22, 1961.
The excellent technical assistance of Mrs. Lillian G. Snyder is gratefully appreciated.
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TABLE I.—HALF-WAVE POTENTIALS, $E_{1/2}$, OF *p*-CHLOROBENZALDOXIME AS A FUNCTION OF HYDROCHLORIC ACID MOLARITY AT 25° IN 10% ETHANOL

$E_{1/2}$	*M* HCl
−0.658	0.05
−0.648	0.075
−0.638	0.10
−0.627	0.15
−0.621	0.20

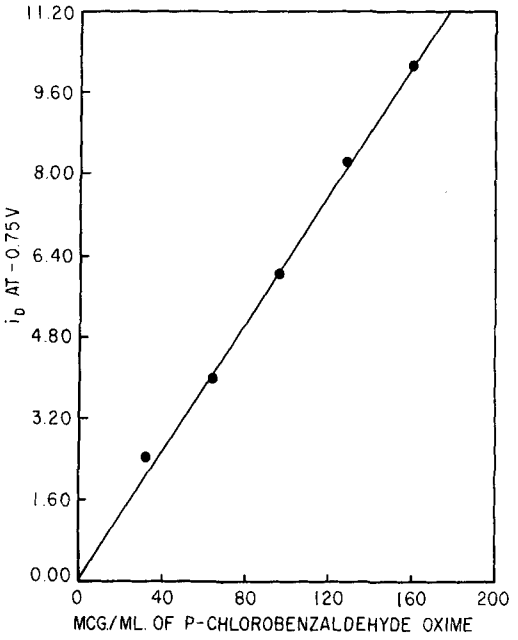


Fig. 2.—Calibration curve for *p*-chlorobenzaldoxime in 0.100 *M* hydrochloric acid, 10% ethanol-water at 25°; diffusion current, i_D in μa., *vs.* concentration in mcg./ml.

benzaldoxime, 0.20 *M* hydrochloric acid, 25°, 10% ethanol. A maximum appeared at −0.68 v. which necessitated the addition of gelatin. The decrease of the wave height characterized by an $E_{1/2}$ of −0.64 is associated with the hydrolysis of the *p*-chlorobenzaldoxime whereas the increase of the wave characterized by an $E_{1/2}$ of −0.84 is associated with the appearance of the hydrolysis product, *p*-chlorobenzaldehyde.

Procedure.—*p*-Chlorobenzaldoxime (PCBO), molecular weight = 155.58, synthesized and recrystallized by G. H. Smith and A. J. Lemin of these laboratories, was titrated and had pKa's of 10.35 in 20% ethanol, 10.70 in 50% ethanol. An 80-mg. quantity of PCBO was weighed into a tared 50-ml. volumetric flask and diluted to volume with nitrogen-purged ethanol. Ten milliliters of the resultant solution was pipetted into a 100-ml. volumetric flask which contained hydrochloric acid plus 2 ml. 0.1% gelatin solution. Subsequently, the solution was diluted up to volume with sufficient hydrochloric acid so that the resultant molarities were 10^{-3} in PCBO, and 0.050, 0.075, 0.150, or 0.200 *M* in hydrochloric acid and 10% in ethanol. The solutions were assayed on the polarograph immediately and at timed intervals thereafter. The solution was maintained in a 25° constant temperature bath between polarographic assays and was degassed with nitrogen for approximately 3 minutes before each run.

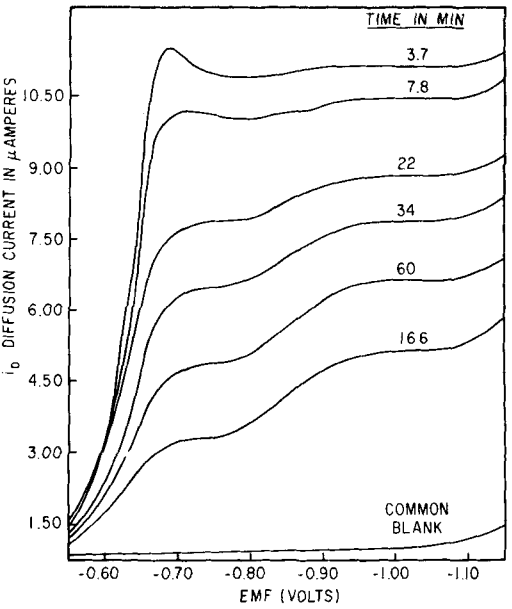


Fig. 3.—Polarographic waves of *p*-chlorobenzaldoxime in 0.200 *M* hydrochloric acid, 10% ethanol-water at 25° at various times.

CALCULATIONS AND RESULTS

The i_D diffusion current at −0.75 and −1.00 v. for the PCBO solutions decreased with time, the latter less than the former since the plateau of the wave representing the *p*-chlorobenzaldehyde concentration appeared prior to −1.00 volts. This wave does not interfere with the −0.75 v. reading. Typical plots of these i_D values as functions of time are given at the top of Fig. 4. The net i_D, i.e., i_D (−1.0 v.) $-i_D$ (−0.75 v.), representing the appearance of the benzaldehyde is given in the bottom of Fig. 4.

The fact that the i_D at −0.75 v. did not approach the i_D of the blank at infinite time indicated that this is an equilibrium reaction.

The plots of Fig. 5 are typical examples on the assumption of an apparent first-order transformation of PCBO to, and in equilibrium with, PCB at constant hydrochloric acid. Curve A represents the first-order disappearance of PCBO by the expression

$$\log [i_D - (i_D)_\infty] = \frac{-(k_1 + k_2)}{2.303} t + \log [(i_D)_0 - (i_D)_\infty] \quad \text{(Eq. 1)}$$

where the i_D values are read at −0.75 v., $(i_D)_0$ is the initial reading, and $(i_D)_\infty$ the reading in μa. at infinite time. Curve B represents the first-order appearance of PCB by the expression

$$\log \{[(i_D)_\infty(-1.0 \text{ v.}) - (i_D)_\infty(-0.75 \text{ v.})] - [i_D(-1.0 \text{ v.}) - i_D(-0.75 \text{ v.})]\} = -\frac{(k_1 + k_2)}{2.303} t + \text{constant} \quad \text{(Eq. 2)}$$

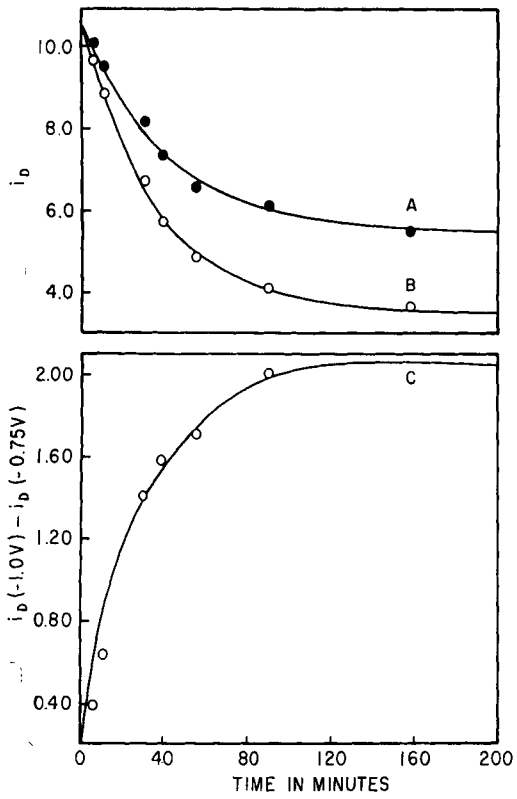


Fig. 4.—Polarographic diffusion current, i_D, of 10^{-3} M p-chlorobenzaldoxime in 0.150 M hydrochloric acid 10% ethanol-water at 25° against time of reading: ○, −0.75 v., ●, −1.00 v.

When it is realized that the data for curve B is obtained from the subtraction of small differences of relatively large values, the empirical use of the assumed model is not unwarranted.

From the slopes of plots similar to curve A, Fig. 5, the sum of the forward and backward rate constants, $k_1 + k_2$, can be calculated as per the postulated equilibrium

$$p\text{-chlorobenzaldoxime} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} p\text{-chlorobenzaldehyde} \qquad \text{(Eq. 3)}$$

where

$$\frac{k_1}{k_2} = \frac{[\text{Benzaldehyde}]}{[\text{Benzaldoxime}]} = K \qquad \text{(Eq. 4)}$$

where K is defined as the equilibrium constant. This K may be estimated from

$$K = \frac{(i_D)_0 - (i_D)_\infty}{(i_D)_\infty} \qquad \text{(Eq. 5)}$$

for all i_D at −0.75 v. From Eqs. 1, 4, and 5; k_1' k_2, and K can be calculated and are given in Table II.

The k_1, k_2, and K values of Table II are plotted in Fig. 6 and dependence on hydrochloric acid concentration may be estimated as

$$k_1\,(\text{min.}^{-1}) = 0.045\,[\text{HCl}] + 0.0134 \qquad \text{(Eq. 6)}$$

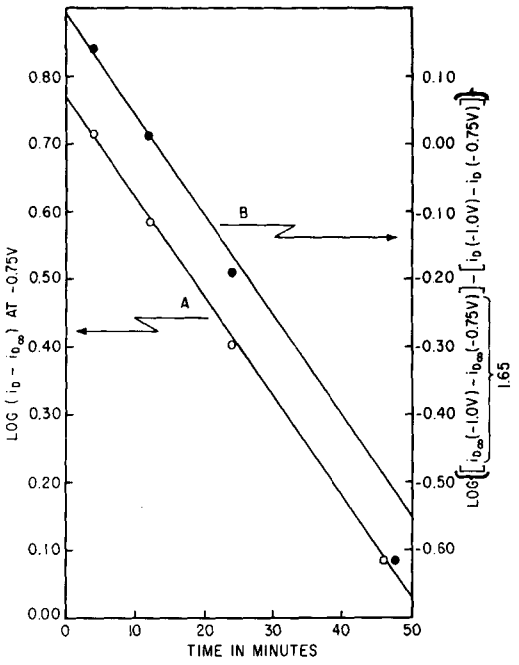


Fig. 5.—Apparent first-order rate plots for the hydrolysis of $10^{-3}M$ p-chlorobenzaldoxime (PCBO) in 0.075 M hydrochloric acid, 10% ethanol-water at 25°. Curve A is for the disappearance of PCBO; curve B is for appearance of p-chlorobenzaldehyde.

TABLE II.—RATES k_1 AND k_2 IN MIN.$^{-1}$ AND EQUILIBRIUM (K) CONSTANTS FOR THE ASSUMED FIRST-ORDER (FORWARD AND REVERSE) TRANSFORMATION OF p-CHLOROBENZALDOXIME $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ p-CHLOROBENZALDEHYDE IN 10% ETHANOL, 25°, $10^{-3}M$ IN PCBO. K' IS WHEN THE BACK REACTION IS ASSUMED SECOND ORDER WITH RESPECT TO PCB AND HYDROXYLAMINE

[HCl]	$k_1 + k_2$	k_1	k_2	K	$10^4K'$
0.050	0.0326	0.0152	0.0174	0.870	4.35
0.075	0.0340	0.0185	0.0155	1.194	6.99
0.150	0.0312	0.0211	0.0102	2.071	15.0
0.200	0.0273	0.0216	0.0057	3.778	32.1

A possible alternate dependency is given by the dashed line. The other estimated constants are

$$k_2\,(\text{min.}^{-1}) = -0.00751\,[\text{HCl}] + 0.021 \qquad \text{(Eq. 7)}$$

$$K = 17.5\,[\text{HCl}] \qquad \text{(Eq. 8)}$$

Actually, a more elegant postulation of the equilibrium would be

$$\text{PCBO} \underset{k_2'}{\overset{k_1}{\rightleftharpoons}} \text{PCB} + \text{Hydroxylamine} \qquad \text{(Eq. 9)}$$

and

$$K' = \frac{[\text{PCB}]\,[\text{Hydroxylamine}]}{[\text{PCBO}]} \qquad \text{(Eq. 10)}$$

where

$$[\text{PCB}] = [\text{Hydroxylamine}] \qquad \text{(Eq. 11)}$$

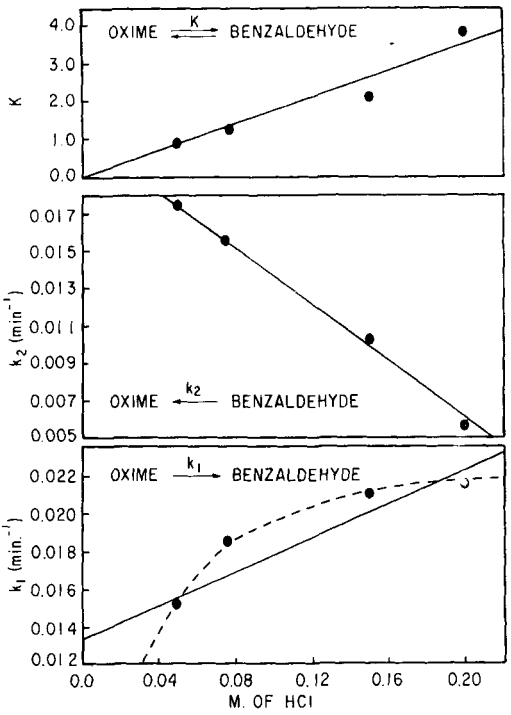


Fig. 6.—Plots of apparent equilibrium constant, K and rate constants, k_1 and k_2, for the simplified equilibrium

$$\text{PCBO} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{PCB}$$

as a function of hydrochloric acid molarity.

so that an equation comparable to Eq. 5 would be

$$K' = \frac{\{(i_D)_0 - (i_D)_\infty \div 10^{-4}\}^2}{(i_D)_\infty \div 10^{-4}} = \frac{\{(i_D)_0 - (i_D)_\infty\}^2 \times 10^4}{(i_D)_\infty} \quad \text{(Eq. 12)}$$

where

$$i_D/C = 10^4\ \mu\text{a.}/M/L. \quad \text{(Eq. 13)}$$

Values for K' are also given in Table II. The plots to determine rate constants could be based on the more elegant and complete expression (4)

$$2.303 \log \frac{[\text{PCBO}]_0 [\text{PCB}]_{eq} + [\text{PCB}]\{[\text{PCBO}]_0 - [\text{PCB}]_{eq}\}}{[\text{PCBO}]_0\{[\text{PCB}]_{eq} - [\text{PCB}]\}} = \frac{kt\{2[\text{PCBO}]_0 - [\text{PCB}]_{eq}\}}{[\text{PCB}]_{eq}} \quad \text{(Eq. 14)}$$

However, since the Eq. 1 satisfactorily fits the data, the simplified assumption of opposing first-order reactions is adequate for the purposes of this study.

DISCUSSION

For our purposes k_1 is given at 25° in 10% ethanol by Eq. 6.

The hydrolysis of PCBO is an equilibrium reaction under the conditions studied. On ingestion into the stomach, however, it is reasonable to postulate the removal of end products PCB and hydroxylamine, so that the hydrolysis tends to be complete.

If the acid concentration of the stomach is assumed to be 0.05 M, the apparent first-order rate constant may be estimated as 0.015 min.$^{-1}$ from Eq. 6 or the half-life for the hydrolysis of the PCB oxime would be

$$t_{1/2} = \frac{2.303 \log 0.5}{k} = \frac{(0.69)}{0.015} = 46 \text{ min.} \quad \text{(Eq. 15)}$$

This is at 25° and in 10% ethanol. It is to be expected that at 37° or body temperature the rate would be at least doubled so that $t_{1/2}$ = 23 min. at 37°. Also, in truly aqueous system the rate should be further increased. Thus, it may be estimated that the half-life of PCBO under the physiological conditions of the human stomach would be less than 20 minutes, and the oral inefficacy of such oximes may be attributed to acid hydrolysis in the stomach.

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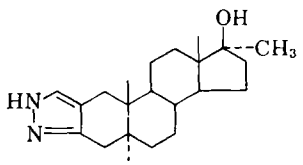
Steroids IX

Synthesis of Some Thiazolosteroids

By NORMAN J. DOORENBOS and CONRAD P. DORN, Jr.†

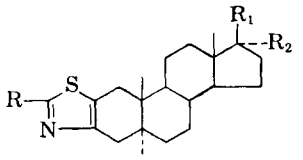
The synthesis of thiazolo[d-3,2]-5α-cholest-2-ene, and thiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol, and eight of their 2′-derivatives is reported. These derivatives include the 2′-methyl, 2′-amino, 2′-allylamino, and 2′-phenylamino substituted thiazolosteroids. Preliminary data indicate that 2′-methylthiazolo[d-3,2]-17α methyl-5α-androst-2-en-17β-ol is a good anabolic agent.

In recent years the search for an effective anabolic agent possessing only low androgenic activity has been markedly intensified. Many modifications of the basic androstane nucleus have been made with moderate success. Recently, however, the Sterling-Winthrop Research Institute announced that a pyrazole derivative, namely, pyrazolo[c-3,2]-17α-methyl-5α-androstan-17β-ol has 35 times the anabolic potency and only one-fourth the androgenic potency of methyltestosterone (1).



The structural features by which this molecule differs from methyltestosterone include: hydrogenation of the Δ^4 double bond with transfusion of rings A and B, a nitrogen atom attached to carbon 3 in place of oxygen, and a heterocyclic ring system fused to ring A between positions 2 and 3. It appears that the above modifications of the steroid nucleus increase the anabolic/androgenic ratio of the compound.

With the above facts in mind, we sought to prepare a series of thiazolosteroids in the cholestane and androstane series. These compounds have the following structure



R_1 = C_8H_{17}; R_2 = H
R_1 = OH; R_2 = CH_3
R = H, CH_3, NH_2, C_6H_5—NH—, CH_2=CH—CH_2—NH—

The cholestane derivatives were prepared as a means of perfecting our synthetic methods before we began working with the more expensive androstane compounds. We were also hopeful that the thiazolocholestanes would have some interesting biological activity, perhaps that of inhibiting cholesterol biosynthesis.

A preliminary report (2) of the thiazoloandrostanes was made following a communication (3) from Syntex describing the independent synthesis of two of these compounds, XI and XII.

The synthesis of thiazole compounds may be accomplished by the method of Hantzsch (4). This method involves the reaction of an α-halo carbonyl compound with a thioamide. By using the proper combination of reactants, compounds may be prepared with alkyl, aryl, or other groups attached to the three carbons of the thiazole nucleus.

We prepared the α-halo carbonyl compound, 2α-bromo-5α-cholestan-3-one (II) according to the method of Fieser and Dominguez (5). This procedure entails the bromination of 5α-cholestan-3-one (I) in glacial acetic acid in the presence of hydrobromic acid to give 2α-bromo-5α-cholestan-3-one (II) in a 44% yield. The carbonyl group of the brominated ketone absorbs at 5.77 μ compared to 5.86 μ in the nonbrominated ketone. This hypsochromic shift indicates that the bromine atom is equatorial and in the 2α-position (6).

The first step in the preparation of the thiazoloandrostanes was the reduction of 17α-methyl-

Received April 28, 1961, from the School of Pharmacy, University of Maryland, Baltimore 1.
Accepted for publication July 19, 1961.
Abstracted from a thesis submitted by Conrad P. Dorn, Jr., to the Graduate Faculty of the University of Maryland, School of Pharmacy, in partial fulfillment of the requirements for the degree of Master of Science.
This work was supported in part by Research Grant CY-4132 from the National Cancer Institute, U. S. Public Health Service.
For paper VIII in this series see Doorenbos, N. J., and Singh, H., This Journal, **50**, 628(1961).
Appreciation is expressed to Smith Kline & French Laboratories for the optical rotational data.
Presented to the Scientific Section, A.Ph.A., Chicago meeting, April 1961.
† American Foundation for Pharmaceutical Education Fellow (1959–1960); National Science Foundation Fellow (1960–1961).

testosterone (VIII). This reaction was carried out in liquid ammonia in the presence of lithium metal according to the procedure which Weisenborn and Applegate (7) used for the reduction of testosterone. This procedure, when used for the reduction of 17α-methyltestosterone (VIII), gave 17α-methyl-5α-androstan-17β-ol-3-one (IX) in a 90% yield. The melting point was in agreement with that previously reported for the 5α-isomer (8).

The bromination of 17α-methyl-5α-androstan-17β-ol-3-one (IX) was accomplished by the use of pyridine hydrobromide perbromide as the brominating agent (9). Tetrahydrofuran was used as the solvent for this reaction to absorb the hydrogen bromide evolved and thus prevent dehydration at position 17. This procedure gave 2α-bromo-17α-methyl-5α-androstan-17β-ol-3-one (X) in a 41% yield. Once again the shift in the carbonyl absorption from 5.85 μ to 5.76 μ indicates that the bromine is equatorial or in the 2α-position.

The substituted thiazole compounds were prepared by refluxing the steroid bromoketones with the appropriate thioamide in ethanol or a mixture of ethanol and chloroform for 3 to 6 hours. Isolation of the free thiazolosteroid was accomplished by treating the hydrobromide salt with base. No difficulty was encountered when the thioamide was thioacetamide, thiourea, N-phenylthiourea, or N-allylthiourea. 2α-Bromo-5α-cholestan-3-one (II) reacted with thioacetamide to yield 22% of 2′-methylthiazolo[d-3,2]-5α-cholest-2-ene (IV); with thiourea to yield 79% of 2′-aminothiazolo[d-3,2]-5α-cholest-2-ene (V); with N-phenylthiourea to yield 45% of 2′-phenylaminothiazolo[d-3,2]-5α-cholest-2-ene (VI); and with N-allylthiourea to yield 50% of 2′-allylaminothiazolo[d-3,2]-5α-cholest-2-ene (VII). Likewise, 2α-bromo-17α-methyl-5α-androstan-17β-ol-3-one (X) reacted with thioacetamide to yield 49% of 2′-methylthiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XII); with thiourea to yield 57% of 2′-aminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIII); with N-phenylthiourea to yield 35% of 2′-phenylaminothiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIV); and with N-allylthiourea to yield 71% of 2′-allylaminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XV).

The preparation of the unsubstituted thiazole derivatives, however, presented a problem. Thioformamide is unstable and must be prepared just prior to use, usually by reacting formamide with phosphorus pentasulfide. We attempted the synthesis of the unsubstituted thiazole in the cholestane series by two methods. The first was a modification of the procedure suggested by Schwartz (10) for the synthesis of 2,4-dimethylthiazole from chloroacetone, acetamide, and phosphorus pentasulfide. We first treated formamide with phosphorus pentasulfide in dry benzene for 1 hour and then added the steroid bromoketone. This procedure gave a product which was extremely difficult to purify. In addition the yield of thiazolo[d-3,2]-5α-cholest-2-ene (III) was only 3.5%.

The method recommended by Cerecedo and Tolpin (11) for the synthesis of thioformamide was then tried. The crude thioformamide was prepared by the addition of phosphorus pentasulfide to formamide which have been covered with anhydrous ether. This mixture was allowed to stand overnight in an ice bath and was then shaken for 15 hours. The ether layer was separated and evaporated at reduced temperature and pressure to yield the crude thioformamide. The thioformamide was added in generous excess to a solution of 2α-bromo-5α-cholestan-3-one (II) in a mixture of chloroform and ethanol. The reaction mixture was stored in the refrigerator for 4 days and allowed to stand at room temperature for 2 weeks. The yield of thiazolo[d-3,2]-5α-cholest-2-ene (III) was 48%. Similarly, 2α-bromo-17α-methyl-5α-androstan-17β-ol-3-one (X) when allowed to react with crude thioformamide for 5 days in the cold and 6 weeks at room temperature yielded 33% of thiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XI).

Proof of the thiazole structures was based on elemental analyses and infrared spectral data. Randall, *et al.* (12), report that thiazoles show characteristic absorption at 6.12–6.37 μ (thiazole I) and 6.50–6.70 μ (thiazole II). All of our compounds with the exception of the methyl thiazoles absorbed in these regions. The methyl compounds had the thiazole II peak at approximately 6.40 μ. In addition, the characteristic absorptions of hydroxyl, amino, phenyl, and allyl groups were observed in the appropriate spectra.

EXPERIMENTAL[1]

Thiazolo[d-3,2]-5α-cholest-2-ene (III).—(*Method A*).—Formamide, 1.8 Gm., and 1.8 Gm. of phosphorus pentasulfide were heated for 1 hour in dry benzene on a steam bath. To this mixture was added a solution of 9.3 Gm. of 2α-bromo-5α-cholestan-3-one (II) dissolved in 100 ml. of dry benzene. The reaction mixture was refluxed 5 hours and filtered

[1] All melting points are uncorrected and were determined with either a Fisher Johns or a Thomas Hoover melting point apparatus. The infrared spectra were prepared on a Perkin Elmer Infracord recording spectrophotometer. The specific rotations were determined by the analytical section of Smith Kline & French Laboratories.

while hot to remove the insoluble impurities. The benzene solution was evaporated to dryness and yielded a yellow-white mass. This material was dissolved in 500 ml. of ethanol and, upon the addition of 2 Gm. of potassium hydroxide, turned orange-red. The solution was heated 30 minutes and the solvent evaporated. Ether was added to the solid material and the ether solution washed well with water. The ether solution was evaporated and the noncrystallizable mass was treated with anhydrous ether saturated with hydrogen bromide in an attempt to isolate the hydrobromide salt. The colloidal precipitate could not be filtered and the ether suspension was evaporated to dryness. The resulting brown mass was washed with dry acetone and yielded a white precipitate. This precipitate was dissolved in ethanol and 2 Gm. of potassium hydroxide was added. The solution was refluxed 30 minutes, cooled, and water added to precipitate the product. This dirty white powder was treated with charcoal in ethanol, filtered while hot, and cooled. The steroid was precipitated by the addition of water. This crude material (m.p. 163–170°) was recrystallized from methanol-water and finally from dimethylformamide to yield 300 mg. (3.5%) of thiazolo[d-3,2]-5α-cholest-2-ene (III) as yellow-white crystals; m.p. 163.5–165°.

Anal.—Calcd. for $C_{28}H_{45}NS$: C, 78.63; H, 10.60; N, 3.27. Found: C, 78.67; H, 10.60; N, 3.09.

(*Method B*).—To 5.0 Gm. of 2α-bromo-5α-cholestan-3-one (II), dissolved in 50 ml. of chloroform-ethanol (1:1), was added 5.0 Gm. of crude thioformamide (11) dissolved in 50 ml. of chloroform-ethanol (1:1). The solution was placed in the refrigerator for 4 days and then allowed to stand at room temperature for 2 weeks. The reaction mixture was made alkaline by the addition of methanolic potassium hydroxide and washed well with water. The organic solution was dried over sodium sulfate and evaporated to dryness. The yellow solid obtained was recrystallized from dimethylformamide-acetone (1:1) to yield 2.2 Gm. (48%) of thiazolo[d-3,2]-5α-cholest-2-ene (III) as fine yellow-white needles; m.p. 166–168°; $[\alpha]_D^{25}$ +55.3° (c, 0.7 chloroform); $\lambda_{max.}^{KBr}$ 6.12 and 6.42 μ.

Anal.—Calcd. for $C_{28}H_{45}NS$: C, 78.63; H, 10.60; N, 3.27. Found: C, 78.72; H, 10.64; N, 3.21.

2′-Methylthiazolo[d-3,2]-5α-cholest-2-ene (IV).—2α-Bromo-5α-cholestan-3-one (II), 2.0 Gm., and 0.5 Gm. of thioacetamide were refluxed 5 hours in 200 ml. of chloroform-ethanol (1:1). The solvent was evaporated and the hydrobromide salt washed with a small amount of ether. The solid material was dissolved in 25 ml. of boiling ethanol. The solution was made alkaline by the addition of potassium hydroxide and refluxed for 30 minutes. The solution was cooled and the precipitated thiazole collected by filtration. The precipitate was washed with 50 ml. of cold acetone and yielded 800 mg. (22%) of 2′-methylthiazolo[d-3,2]-5α-cholest-2-ene (IV) as a white powder; m.p. 168.5–169.5°; $[\alpha]_D^{25}$ +52.9° (c, 0.81 chloroform); $\lambda_{max.}^{KBr}$ 6.12 and 6.41 μ.

Anal.—Calcd. for $C_{29}H_{47}NS$: C, 78.84; H, 10.73; N, 3.17. Found: C, 78.58; H, 11.02; N, 3.21.

2′-Aminothiazolo[d-3,2]-5α-cholest-2-ene (V).—2α-Bromo-5α-cholestan-3-one (II), 2.0 Gm., and 5.0 Gm. of thiourea were refluxed for 5 hours in 75 ml. of chloroform-ethanol (1:1). A white precipitate formed after about 1 hour. The reaction mixture was evaporated to dryness. The solid residue was redissolved in 250 ml. of ethanol and made alkaline with potassium hydroxide. The solution was cooled and the precipitate collected by filtration. The crude thiazolosteroid was recrystallized from ethanol to yield 1.5 Gm. (79%) of 2′-aminothiazolo-[d-3,2]-5α-cholest-2-ene (V) as fluffy white crystals; m.p. 271–271.5°; $[\alpha]_D^{25}$ +58.3° (c, 0.78 chloroform); $\lambda_{max.}^{KBr}$ 2.82–3.02, 3.13–3.25, 6.10, and 6.53 μ [reported m.p. 266°; $[\alpha]_D^{25}$ +64.4° (c, 0.49 chloroform)] (13).

Anal.—Calcd. for $C_{28}H_{46}N_2S$: C, 75.97; H, 10.47; N, 6.32. Found: C, 75.88; H, 10.47; N, 6.32.

2′-Phenylaminothiazolo[d-3,2]-5α-cholest-2-ene (VI).—2α-Bromo-5α-cholestan-3-one (II), 4.0 Gm., and 1.5 Gm. of N-phenylthiourea were refluxed for 6 hours in 400 ml. of ethanol The reaction mixture was made alkaline with methanolic potassium hydroxide and concentrated until crystals began to appear. The solution was cooled and the precipitate collected. The crude product was recrystallized from ethanol to yield 2.0 Gm. (45%) of 2′-phenylaminothiazolo[d-3,2]-5α-cholest-2-ene (VI) as white needles; m.p. 194.5–195.5°; $[\alpha]_D^{25}$ +62.9° (c, 0.99 chloroform); $\lambda_{max.}^{CHCl_3}$ 2.93, 6.21, 6.50, and 6.67 μ.

Anal.—Calcd. for $C_{34}H_{50}N_2S$: C, 78.70; H, 9.71; N, 5.40. Found: C, 78.68; H, 9.47; N, 5.34.

2′-Allylaminothiazolo[d-3,2]-5α-cholest-2-ene (VII).—2α-Bromo-5α-cholestan-3-one (II), 5.0 Gm., and 2.0 Gm. of N-allylthiourea were refluxed 3 hours in ethanol. The solution was made alkaline with methanolic potassium hydroxide, concentrated, and the precipitate collected by filtration. The crude product was recrystallized from chloroform-methanol to give 2.6 Gm. (50%) of 2′-allylaminothiazolo-[d-3,2]-5α-cholest-2-ene (VII) as white granules; m.p. 189.5–192°; $[\alpha]_D^{25}$ +57.4° (c, 1.03 chloroform); $\lambda_{max.}^{CHCl_3}$ 2.92, 6.07, 6.30, and 6.49 μ.

Anal.—Calcd. for $C_{31}H_{50}N_2S$: C, 77.13; H, 10.44; N, 5.80. Found: C, 76.63; H, 10.30; N, 5.91.

17α-Methyl-5α-androstan-17β-ol-3-one (IX).—17α-Methyltestosterone (VIII), 5.0 Gm., was dissolved in 120 ml. of 50:50 dry dioxane-ether. This solution was added, dropwise, with stirring to a solution of 1.0 Gm. of lithium metal in approximately 500 ml. of liquid ammonia. Thirty minutes after the addition was completed the blue color of the ammonia solution was discharged by the cautious addition of ammonium chloride. The ammonia was evaporated and the solid material taken up in chloroform and washed well with water. The chloroform solution was dried over sodium sulfate and evaporated to dryness. The residue was crystallized from methanol to yield 4.5 Gm. (90%) of 17α-methyl-5α-androstan-17β-ol-3-one (IX) as white granular crystals; m.p. 192–194°; $\lambda_{max.}^{CHCl_3}$ 2.76, and 5.85 μ (reported m.p. 193°) (8).

2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X).—17α-Methyl-5α-androstan-17β-ol-3-one (IX), 6.0 Gm., was dissolved in 500 ml. of tetrahydrofuran. To this solution was added, portionwise, 6.4 Gm. of pyridine hydrobromide perbromide. The mixture was allowed to stand overnight at room temperature and then poured into 200 ml. of water. This mixture was extracted well with ether and the ether solution dried over sodium sulfate and evaporated to dryness. The residue was crystallized from ethyl acetate-petroleum ether to yield 3.1 Gm. (41%)

of 2α-bromo-17α-methyl-5α-androstan-17β-ol-3-one (X) as tan crystals; m.p. 185–187°; $\lambda_{max.}^{CHCl_3}$ 2.76 and 5.76 μ (reported m.p. 196–198°) (3).

Anal.—Calcd. for $C_{20}H_{31}BrO_2$: C, 62.66; H, 8.15; Br, 20.84. Found: C, 62.54; H, 8.14; Br, 21.30.

Thiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XI).—2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X), 5.0 Gm., was dissolved in 100 ml. of chloroform. To this solution was added 10 Gm. of crude thioformamide (11) dissolved in 50 ml. of chloroform-ethanol (1:1). The reaction mixture was stored in the refrigerator for 5 days and then allowed to stand at room temperature for 6 weeks. The reaction mixture was then made alkaline and washed well with water. The chloroform solution was dried over sodium sulfate and evaporated to yield a dark oil. The oil was dissolved in a minimum of dimethylformamide and allowed to stand at room temperature. After a week, oily crystals appeared and were collected by filtration. The crude material was recrystallized from anhydrous ether-petroleum ether to yield 1.5 Gm. (33%) of thiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XI) as salmon colored granules; m.p. 177–179°; $[\alpha]_D$ +48.8°; $\lambda_{max.}^{EtOH}$ 251 mμ, log ε 3.64; $\lambda_{max.}^{CHCl_3}$ 2.75, 3.00–3.05, and 6.40 μ (reported m.p. 192–193°; $[\alpha]_D$ +33°; $\lambda_{max.}^{EtOH}$ 252 mμ, log ε 3.61) (3).

Anal.—Calcd. for $C_{21}H_{31}NOS$: C, 73.02; H, 9.04; S, 9.53. Found: C, 72.66; H, 9.01; S, 9.53.

2′-Methylthiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XII).—2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X), 1.5 Gm., and 1.5 Gm. of thioacetamide were refluxed 5 hours in ethanol. The reaction mixture was made alkaline with methanolic potassium hydroxide. Ether (150 ml.) was added and the resulting solution washed well with water. The organic layer was dried over sodium sulfate and evaporated to dryness. The crude material was recrystallized twice from acetone to yield 700 mg. (49%) of 2′-methylthiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XII) as yellow-white needles; m.p. 198–199.5°; $[\alpha]_D$ +35.6°; $\lambda_{max.}^{EtOH}$ 253 mμ, log ε 3.72; $\lambda_{max.}^{CHCl_3}$ 2.76, and 6.40 μ (reported m.p. 212–215°; $[\alpha]_D$ +47°; $\lambda_{max.}^{EtOH}$ 254 mμ, log ε 3.76) (3).

Anal.—Calcd. for $C_{22}H_{33}NOS$: C, 73.48; H, 9.25; N, 3.90; S, 8.92. Found: C, 73.42; H, 9.23; N, 4.12; S, 9.04.

2′-Aminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIII).—2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X), 5.0 Gm., and 3.0 Gm. of thiourea were refluxed 5 hours in ethanol. The reaction mixture was then made alkaline with methanolic poatassium hydroxide and concentrated until the crude product precipitated. This precipitate was filtered and recrystallized from methanol-water to yield 2.7 Gm. (57%) of 2′-aminothiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIII) as white plates; m.p. 270–272°; $[\alpha]_D$ +45.0°; $\lambda_{max.}^{CHCl_3}$ 2.76, 2.86, 2.94, 6.25, and 6.57 μ.

Anal.—Calcd. for $C_{21}H_{32}N_2OS$: C, 69.95; H, 8.95; N, 7.77. Found: C, 69.80; H, 9.26; N, 7.47.

2′-Phenylaminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIV).—2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X), 3.0 Gm., and 3.0 Gm. of N-phenylthiourea were refluxed 3 hours in ethanol. The reaction mixture was made alkaline with methanolic potassium hydroxide and concentrated until the crude product precipitated. This material was filtered and recrystallized from ethanol to yield 1.2 Gm. (35%) of 2′-phenylaminothiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XIV) as white plates; m.p. 250–252°; $[\alpha]_D$ +46.0°; $\lambda_{max.}^{EtOH}$ 298 mμ, log ε 4.21; $\lambda_{max.}^{KBr}$ 2.76, 2.90, 2.06, 6.19, 6.48, and 6.64 μ.

Anal.—Calcd. for $C_{27}H_{36}N_2OS$: C, 74.27; H, 8.31; N, 6.42. Found: C, 74.61; H, 8.41; N, 6.14.

2′-Allylaminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XV).—2α-Bromo-17α-methyl-5α-androstan-17β-ol-3-one (X), 5.0 Gm. and 2.0 Gm. of N-allylthiourea were refluxed 3 hours in ethanol. The reaction mixture was made alkaline by the addition of methanolic potassium hydroxide and concentrated until the crude product precipitated. This material was filtered and recrystallized from ethanol-water to yield 3.7 Gm. (71%) of 2′-allylaminothiazolo[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (XV) as white needles; m.p. 232–234°; $[\alpha]_D$ +50.5°; $\lambda_{max.}^{EtOH}$ 266 mμ, log ε 3.93; $\lambda_{max.}^{CHCl_3}$ 2.76, 2.90, 6.07, 6.27, and 6.49 μ.

Anal.—Calcd. for $C_{24}H_{36}N_2OS$: C, 71.96; H, 9.06; N, 6.99. Found: C, 71.96; H, 9.13; N, 7.15.

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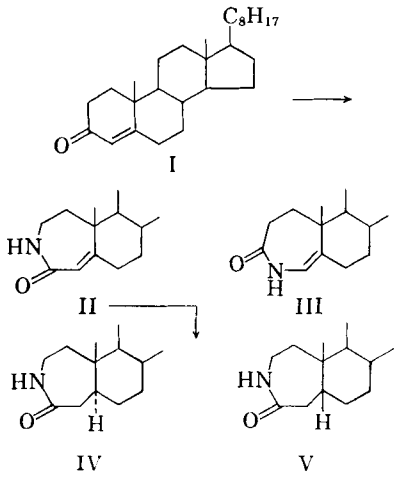
Steroids X

Preparation of Azasteroids from Cholestenone and Progesterone

By NORMAN J. DOORENBOS and HARKISHAN SINGH†

Cholestenone (I) was converted into 3-aza-A-homo-4a-cholesten-4-one (II) by means of the Schmidt reaction in polyphosphoric acid. The structure was assigned on the basis of its ultraviolet spectrum, resistance to hydrolysis, and hydrogenation to a mixture of 3-aza-A-homo-5α-cholestan-4-one (IV) and 3-aza-A-homo-5β-cholestan-4-one (V). By similar procedures, progesterone (VI) yielded either 17β-acetylamino-4-androsten-3-one (VII) or 17β-acetylamino-3-aza-A-homo-4a-androsten-4-one (VIII), depending upon the quantity of sodium azide used. The structure of VII was confirmed by preparing it from 17β-acetylamino-5-androsten-3β-ol (IX) by Oppenauer oxidation.

THE ANDROGENIC, progestational, and corticosteroid hormones each contain an α,β-unsaturated ketone system in ring A. As an extension of our investigation of azasteroids, we sought to synthesize A-homosteroid homologs of some of these steroids with a nitrogen in position 3 by means of the Schmidt reaction. An example of such a steroid is illustrated by structure II.



The initial studies were carried out with cholestenone (4-cholesten-3-one) (I). No crystalline products were isolated when the Schmidt reaction was carried out by the usual procedures with sulfuric acid as the catalyst (1). However, when polyphosphoric acid was used as the solvent and catalyst (2, 3), 3-aza-A-homo-4a-cholesten-4-one (II) was obtained as a white crystalline product. Chromatographic studies on II established that it was a single compound and not a mixture. Structure II was assigned to this product (*a*) because of its resistance to hydrolysis, (*b*) since its spectrum resembles that of a known steroidal α,β-unsaturated lactam, and differs from known steroidal enamine lactams, and (*c*) because it is hydrogenated to a mixture of 3-aza-A-homo-5α-cholestan-4-one (IV) and 3-aza-A-homo-5β-cholestan-4-one (V).

Enamine lactams of the type represented by structure III are relatively easy to hydrolyze as illustrated by the ease of hydrolysis of 3β-acetoxy-17-acetylamino-5,16-androstadiene (4) and 4-aza-5-cholesten-3-one (5). The product obtained by the Schmidt reaction on cholestenone was not hydrolyzed by refluxing in 5 per cent ethanolic potassium hydroxide or by refluxing in dioxane-concentrated hydrochloric acid mixture. This resistance to hydrolysis indicates that it is the α,β-unsaturated lactam represented by structure II.

Table I lists the major absorption peak in the ultraviolet spectrum for several enamine and α,β-unsaturated steroid lactams. The first four compounds listed are enamine lactams. The fifth is an α,β-unsaturated lactam. The spectrum of lactam II, the sixth compound in this list, is similar to that of the α,β-unsaturated lactam.

This unsaturated lactam (II) was reduced with platinum and hydrogen in glacial acetic acid at 70° and 60 lb. pressure. A reduced lactam was separated from some unreacted starting material by chromatography. This reduced lactam, m.p. 202–245°, was a mixture of the 5α-[m.p. 268–271° (6)] and 5β-[m.p. 166–174° (6)] isomers. Repeated crystallization from absolute ethanol yielded a sample, m.p. 263–269°, which was shown, by mixed melting points and a comparison of infrared spectra, to be 3-aza-A-homo-5α-cholestan-4-one (IV).

It should be noted that the α,β-unsaturated steroid lactam described in Table I was also

Received April 28, 1961, from the School of Pharmacy, University of Maryland, Baltimore 1.
Accepted for publication July 19, 1961.
This work was supported by Research Grant CY-4132 from the National Cancer Institute, U. S. Public Health Service.
For paper IX in this series see Doorenbos, N. J., and Dorn, C. P., Jr., THIS JOURNAL, 51, 414 (1962).
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
† On a leave of absence from the Department of Pharmacy University of Saugar, M. P., India.

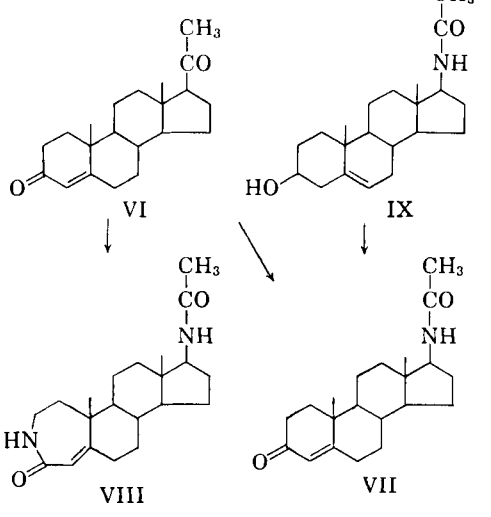
TABLE I.—MAJOR ABSORPTION PEAKS OF SOME UNSATURATED STEROID LACTAMS

Compound	Solvent	$\lambda_{max.}$	Log ϵ
2β-Acetoxy-17-acetylamino-5,16-androstadiene (4)	EtOH	240 mμ	3.82
4-Aza-5-cholesten-3-one (7)	EtOH	233 mμ	4.14
6-Aza-4-cholesten-7-one (8)	EtOH	234 mμ	4.11
4-Aza-4-methyl-5-cholesten-3-one (9)	EtOH	234 mμ	4.13
3β-Acetoxy-12a-aza-C-homo-5α,22α-spirost-9-(11)-en-12-one (10, 11)	MeOH	220 mμ	4.20
3-Aza-A-homo-4a-cholesten-4-one (II)	EtOH	220 mμ	4.05
17β-Acetylamino-3-aza-A-homo-4a-androsten-4-one (VIII)	EtOH	220 mμ	4.14

prepared from an α,β-unsaturated ketone. Mazur (10) prepared this compound from 3β-acetoxy-5α,22α-spirost-9(11)-en-12-one by means of the Beckmann rearrangement of its oxime. The structure which Mazur tentatively assigned to the product was confirmed by Bladon and McMeekin (11).

The Schmidt reaction was carried out with progesterone (VII) under conditions similar to those used for cholestenone (I). 17β-Acetylamino-4-androsten-3-one (VII) was obtained with 1 mole of sodium azide and 17β-acetylamino-3-aza-A-homo-4a-androsten-4-one (VIII) with an excess of sodium azide. The structural assignments were made on the basis of analysis and spectra.

Compound VII (12, 13) was prepared by Oppenauer oxidation of 17β-acetylamino-5-androsten-3β-ol (IX) (14) and shown by means of mixed melting points and a comparison of spectra to be identical with the sample prepared by the Schmidt reaction of progesterone.



EXPERIMENTAL[1]

3-Aza-A-homo-4a-cholesten-4-one (II).—A mixture of 5.0 Gm. of 4-cholesten-3-one (I), 160 Gm. of polyphosphoric acid, and 1.0 Gm. of sodium azide was heated at 50–60° for 10 hours with frequent stirring, following a previously described procedure for the Schmidt reaction of steroids in polyphosphoric acid (3). The mixture was poured onto crushed ice, made alkaline with cold 50% potassium hydroxide, extracted with chloroform, and washed with water. Removal of the solvent, after drying over sodium sulfate, yielded 5.1 Gm. of a brown solid residue. The residue was chromatographed with 60 Gm. of neutral Woelm alumina, activity grade I, which had been prepared with benzene. Elution with benzene (3 × 150 ml.) yielded a sticky material which melted completely by 63°. A series of elutions extending from chloroform-benzene (1:19) through chloroform yielded 3.1 Gm. (60%), m.p. 220–240°. An analytical sample was prepared by crystallization from benzene-acetone; m.p. 255–260°; $[\alpha]_D^{27}$ +20.0° (c, 1.0 $CHCl_3$); $\lambda_{max.}^{EtOH}$ 220 mμ, log ε 4.05; $\lambda_{max.}^{CHCl_3}$ 291 mμ (free NH); 6.05 μ (lactam C=O); 6.22 μ (C=C).

Anal.—Calcd. for $C_{27}H_{45}NO$: C, 81.14; H, 11.35; N, 3.51. Found: C, 80.89; H, 11.40; N, 3.70.

During the course of subsequent preparations it was established that the product of this reaction could be purified by repeated digestion with petroleum ether.

The initial attempts to prepare this product by the Schmidt reaction using chloroform as the solvent and sulfuric acid as the catalyst yielded an oily product which could not be separated by chromatography.

Attempted Hydrolysis of 3-Aza-A-homo-4a-cholesten-4-one (II).—A 1-Gm. sample of II was added to 100 ml. of 5% potassium hydroxide in 95% ethanol and the mixture was refluxed 1½ hours. The lactam was recovered unchanged.

A mixture of 1 Gm. of II, 150 ml. of dioxane, and 50 ml. of concentrated hydrochloric acid was refluxed 3 hours. The lactam was recovered unchanged.

Hydrogenation of 3-Aza-A-homo-4a-cholesten-4-one (II).—3-Aza-A-homo-4a-cholesten-4-one, 4.0 Gm., was dissolved in 200 ml. of glacial acetic acid. Platinum oxide, 700 mg , was added and the mixture was shaken in a Parr hydrogenator at 72° with a hydrogen pressure of 60 lb. for 9 hours. The mixture was cooled, filtered, diluted, and saturated with sodium chloride. The mixture was extracted with methylene chloride. The extracts were washed with sodium carbonate solution and water and then dried over sodium sulfate. The residue, 3.8 Gm., obtained by evaporating the solvent was chromatographed on a column prepared with 125 Gm. of neutral Woelm alumina, activity grade I. The column was prepared with benzene. No product was obtained by elution with benzene or methylene chloride. Elution with absolute ethanol-methylene chloride, 1:99, (6 × 500 ml.) yielded 2.6 Gm. (frac-

[1] Melting points are uncorrected. Analyses were obtained from Drs. Weiler and Strauss, Oxford, England. Spectra were obtained on Perkin-Elmer Infracord and Spectracord spectrophotometers. The authors wish to express their thanks to the Cancer Chemotherapy National Service Center for the steroid intermediates used in this investigation.

tion A), m.p. 202–245°. There was no absorption which could be attributed to an olefinic double bond. A series of fractions were combined as fraction A since they had similar melting points and identical infrared spectra.

Repeated crystallizations from absolute ethanol raised the melting point to 263–269° without changing the infrared spectrum. A mixed melting point with an authentic sample of 3-aza-A-homo-5α-cholestan-4-one, m.p. 270–272°, gave a m.p. of 264–269°, establishing that these samples are identical.

Further elution of the column with ethanol yielded 800 mg. of starting material, II.

17β-Acetylamino-4-androsten-3-one (VII).—*Method A, from Progesterone.*—Progesterone (VI), 2.0 Gm., was allowed to react with 0.4 Gm. of sodium azide in polyphosphoric acid in a procedure similar to that used for cholestenone. After repeated crystallizations from acetone the crude product yielded 220 mg. of an analytical sample of 17β-acetylamino-4-androsten-3-one (VII), m.p. 275–278°; $[\alpha]_D^{27}$ + 58.0° (c, 1.0 $CHCl_3$); $\lambda_{max.}^{EtOH}$ 239 mμ, log ε 4.11; $\lambda_{max.}^{CHCl_3}$ 2.92 μ (free NH), 3.00 μ (associated NH), 6.01 μ (C=O), and 6.20 μ (C=C). A second crop (500 mg.) was obtained giving a total yield of 720 mg. (35%).

Anal.—Calcd. for $C_{21}H_{31}NO_2$: C, 76.55; H, 9.48; N, 4.25. Found: C, 76.42; H, 9.39; N, 4.69.

Method B, from 17β-Acetylamino-5-androsten-3β-ol (IX).—To a refluxing suspension of 681 mg. of 17β-acetylamino-5-androsten-3β-ol (IX) (14) in a mixture of 100 ml. of dry benzene and 40 ml. of acetone, was added a solution of 4.1 Gm. of aluminum isopropoxide in 20 ml. of benzene. The mixture was refluxed 44 hours. After cooling, 10 ml. of water was added and the mixture was shaken vigorously. Then 40 ml. of dilute sulfuric acid was added and the mixture was again shaken. The benzene layer was separated, washed with water, and dried over sodium sulfate. The residue obtained by evaporating the solvent was crystallized from acetone to yield 345 mg. of 17β-acetylamino-4-androsten-3-one, m.p. 272–277° [reported 270° (12) and 284–286° (13)]. This product was shown to be identical to that prepared by method A by a comparison of infrared spectra and a mixed melting point.

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Glutathione, a sulfhydryl-containing tripeptide, has been implicated as playing some role in conditions of mental aberration. The levels of NPSH detected by the amperometric titration

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procedure employed in this investigation consists mainly of reduced glutathione (3). Lowered blood glutathione levels in schizophrenic patients have been reported (4–6) and another report indicated that glutathione was useful in the treatment of schizophrenic states (7). Other workers have been unable to confirm these results. This investigation was undertaken to study further the effects of sciatic nerve stimulation on brain NPSH and to study the effects of chlorpromazine,[1] and meprobamate,[2] morphine, and meperidine on increased cerebral NPSH levels produced by sciatic nerve stimulation.

EXPERIMENTAL

Holtzman albino rats of both sexes weighing 150–250 Gm. were curarized, rather than pentobarbitalized, with 0.02 mg. of dimethyl tubocurarine iodide per rat and artificially respired during the course of the stimulation. The sciatic nerve was stimulated for 20 minutes with a Grass model S4E stimulator with square wave pulses of 40 volts intensity, 3 milliseconds duration at a frequency of 100 cycles per second. After the nerve was stimulated the rat was sacrificed by decapitation. The brain was removed, washed immediately with cold 2% sulfosalicylic acid, the cerebrum removed and frozen with dry ice. The tissue was weighed and homogenized in cold 2% sulfosalicylic acid, centrifuged, and filtered through shark skin filter paper. The filtrate was diluted to a volume in which 2 ml. represented the extract of 100 mg. of tissue. Two-milliliter aliquots were assayed for NPSH by a modification of the amperometric titration procedure of Benesch and Benesch (8). Duplicate titrations of each sample were made, the results averaged and expressed as micromoles per cent of NPSH.

Control rats were curarized and artificially respired for 20 minutes with the electrode placed on the sciatic nerve, but without stimulation.

With the exception of the meprobamate suspension, all drug solutions were prepared daily. Meprobamate was prepared as an 8% suspension with 4% acacia and 0.1% sodium benzoate. All compounds were administered intraperitoneally. Chlorpromazine hydrochloride was administered 4 hours and meprobamate 2 hours prior to stimulation in doses of 10 mg./Kg. and 275 mg./Kg., respectively. Morphine sulfate and meperidine hydrochloride were administered in doses of 15 mg./Kg. and 30 mg./Kg., respectively. Sciatic stimulation was begun 15 minutes later.

The instability of tissue NPSH, even when frozen, necessitated rapid analysis of tissue samples. All frozen samples were assayed for NPSH within 8 hours after removal. Since the experimental procedures were time consuming, a design was employed in which each experimental group was completed as a separate study. An equal number of control animals was used for each experimental group. Nine rats were used for each treatment in the morphine experiment, while 6 rats were used for each treatment in the three remaining experimental groups.

TABLE I.—EFFECTS OF DRUGS ON SCIATIC STIMULATED CEREBRAL NPSH LEVELS

Treatment	Cerebral NPSH μm. % ± S.E.
Control	158.0 ± 1.5
Sciatic stimulation	205.0 ± 5.5
Stimulation plus chlorpromazine 10 mg./Kg. (4 hr. pretreatment)	161.0 ± 4.0
Chlorpromazine control 10 mg./Kg. (4 hr. pretreatment)	160.8 ± 2.3
Control	156.6 ± 1.0
Sciatic stimulation	182.0 ± 4.0
Stimulation plus meprobamate 275 mg./Kg. (2 hr. pretreatment)	152.5 ± 1.5
Meprobamate control 275 mg./Kg. (2 hr. pretreatment)	157.3 ± 2.0
Control	131.0 ± 5.0
Sciatic stimulation	171.5 ± 7.0
Stimulation plus morphine 15 mg./Kg. (15 min. pretreatment)	132.0 ± 7.0
Morphine control 15 mg./Kg. (15 min. pretreatment)	135.0 ± 9.0
Control	162.5 ± 5.5
Sciatic stimulation	210.5 ± 4.0
Stimulation plus meperidine 30 mg./Kg. (15 min. pretreatment)	168.0 ± 5.0
Meperidine control 30 mg./Kg. (15 min. pretreatment)	167.5 ± 2.5

RESULTS

The results of this investigation are summarized in Table I.

Cerebral NPSH levels of sciatic stimulated rats were significantly greater than those of control rats in each of the four experimental groups. The overall mean increase in cerebral NPSH after sciatic stimulation was 22%. When the applied voltage was reduced from 40 to 4 or less volts, cerebral levels of NPSH were still increased. In an experiment in which 4 rats were subjected to sciatic stimulation with 5 volts, an increase of 12.7% over control rats was noted.

In preliminary experiments, pretreatment with 10 mg./Kg. of chlorpromazine 10 minutes prior to sciatic stimulation was not effective in preventing the rise of cerebral NPSH. These rats were deeply sedated and had lost their righting reflexes. A longer pretreatment period of 4 hours was used before sciatic stimulation. The rats were beginning to regain their righting reflexes before stimulation was begun. As illustrated in Table I, this latter treatment with chlorpromazine completely blocked the rise in cerebral NPSH after sciatic stimulation. Chlorpromazine alone had no effect on cerebral NPSH levels in nonstimulated rats.

Bradley, *et al.* (9) had reported that pretreatment with 400 mg./Kg. of meprobamate intraperitoneally 20 minutes before stimulation was ineffective in preventing the rise of cerebral NPSH. In this study, a 275 mg./Kg. dose of meprobamate given 2 hours prior to stimulation blocked the NPSH rise, as did chlorpromazine. Meprobamate itself had no effect on the NPSH levels of the nonstimulated brain.

[1] Generously supplied by Smith Kline and French Laboratories, Philadelphia, Pa.

[2] Generously supplied by Wallace Laboratories, New Brunswick, N. J.

Morphine and meperidine-treated rats exhibited analgesia at the time stimulation was begun, and retained their righting reflexes. The results obtained were almost identical as those obtained with the meprobamate and chlorpromazine-treated rats. Both drugs prevented the NPSH rise in stimulated rats and neither affected NPSH levels of nonstimulated rats.

DISCUSSION

Ungar and Romano (1) found an increase of 55% in brain protein-bound sulfhydryl and a nonsignificant rise of 29% in dialyzable sulfhydryl in sciatic stimulated rats. In our studies, the 22% increase in NPSH observed was highly significant ($t_{10} = 6.3$; $P < 0.01$). The coefficients of variation in our studies were only about 4%, whereas in their data they were 12 and 16%. The fact that the dialyzable sulfhydryl was estimated by difference may account for the greater variability. It is possible that dialyzable sulfhydryl is not equivalent to the NPSH remaining after protein precipitation with sulfosalicylic acid. Another factor to be considered is that only cerebral NPSH levels were measured in our studies since NPSH of the cerebellum, medulla, and midbrain did not rise after sciatic stimulation.

Ungar and Romano postulated that the increased levels of protein-bound sulfhydryl after stimulation resulted from structural rearrangement of proteins participating in the mechanism of excitation. If NPSH changes parallel protein-bound sulfhydryl changes, then one possible explanation of the blockade of the NPSH rise by morphine, meperidine, chlorpromazine, and meprobamate is that these drugs may have prevented the excitation of the cerebrum, perhaps by a blockade of the neuronal paths from the sciatic nerve. One would have to assume, then, that pentobarbital, which did not block the NPSH rise, is relatively ineffective in preventing excitation of the cerebrum. Whether blockade of neuronal paths occurs can be confirmed by the use of electrophysiological approaches.

The biological roles of glutathione have not been established with certainty. Krimsky and Racker (10) have found glutathione to be a firmly bound prosthetic group of the glycolytic enzyme phosphoglyceraldehyde dehydrogenase. Glutathione reductase has been identified (11) and found to catalyze the oxidation of reduced triphosphopyridine nucleotide in the presence of oxidized glutathione with the resulting formation of reduced glutathione.

Szent-Gyorgyi (12) has suggested that glutathione and ascorbic acid, with their respective enzymes, might form a respiratory chain. The reduced and oxidized forms of these substances form reversible redox systems, and are found in highest concentrations in tissues with the highest metabolic activity. Mapson and Moustafa (13) constructed a model respiratory system in which oxygen consumption fell when levels of reduced glutathione decreased and oxidized glutathione increased. They proposed that oxidized glutathione is reduced by reduced triphosphopyridine nucleotide in the presence of glutathione reductase. The reduced glutathione is then oxidized by dehydroascorbic acid in the presence of dehydroascorbic acid reductase. This involvement of glutathione in carbohydrate metabolism suggests that the rise in cerebral glutathione may possibly be a consequence of accelerated cerebral metabolism which occurs when the sciatic nerve is stimulated.

The psychotropic agents employed in this investigation had no effect on nonstimulated cerebral NPSH levels and, as has been previously shown in this laboratory (9), behavioral changes induced by psychomimetic agents are not reflected by changes in cerebral NPSH. The tranquilizers as well as the analgetics employed in this study blocked the NPSH rise produced by sciatic stimulation. It is interesting to note that the analgetics used block the conditioned avoidance response. It is apparent that induced rises in cerebral NPSH levels are blocked by only certain types of central nervous system depressants since pentobarbitalized animals respond to the sciatic stimulation. Also, loss of consciousness is not essential to the blocking effect. Further work is contemplated to elucidate the mechanism of this blocking action.

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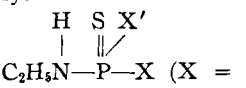
Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters I

Synthesis

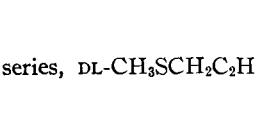
By FLORENCE C. KLEE† and ERNST R. KIRCH

Thiophosphoric acid derivatives having structures similar to known anticholinesterases, were synthesized for the investigation of their possible enzyme inhibition *in vivo*. Based on theoretical considerations concerning structures of known chemotherapeutic agents, these compounds may possess antitumor activity. The phenyl ester was found to be white needle-like crystals rather than the liquid reported by Michaelis. Procedures for obtaining good yields of the ethyl esters of L-proline and DL-methionine are given. A modified method of King's colorimetric determination of phosphorus is presented.

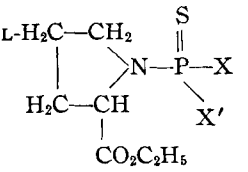
THE PURPOSE of this investigation was to prepare some thiophosphoric acid derivatives which have structures similar to known anticholinesterases, and to investigate their biological action *in vivo*. The three series of compounds synthesized are the following: ethylamine series,



C_2H_5N—P—X (X = X′ = Cl; X = X′ = OR where R is an ethyl, *n*-propyl, isopropyl, *n*-, *sec*-, *tert*- or iso-butyl, or a phenyl group; X = OC_2H_5, X′ = Cl); DL-methionine ethyl ester



series, DL-$CH_3SCH_2C_2H_5O_2C$—P—X, and L-proline ethyl ester series,



where

X = X′ = OC_2H_5

X = X′ = Cl

X = OC_2H_5, X′ = Cl.

These derivatives were selected with the intention of synthesizing anticholinesterases with potential antitumor activity. The rationale for the synthesis of these derivatives is as follows: (*a*) The phosphate radical is very important in biological systems and these organothiophosphorus derivatives may interfere with metabolic pathways providing the energy for cell metabolism of cancer cells. There is a possibility that the P=S group is converted to P=O *in vivo*, but this conversion would not be undesirable, since the anticholinesterase activity of these compounds would be enhanced (1).

(*b*) The amino acids, methionine and proline, were bound to the thiophosphorus radical in an attempt to produce potential amino acid antagonists since the amino acid antagonists DL-ethionine (2) and the bis-chloroethyl derivative of carbamoylserine (3) have been reported as tumor inhibitors. S-Carbamylcysteine has been observed to exhibit antitumor action (4). Methionine has been reported to reduce the growth of a rat sarcoma (5). Certain derivatives of proline have insecticidal activity (6).

(*c*) Certain phosphoric acid ester amides (7) and certain ethylenephosphoramides (8) have carcinostatic activity. A thiophosphoramide (thio-TEPA) (9, 10) is said to be curative and widely used.

(*d*) In the literature, anticholinesterase activity by alkyl phosphate compounds (11 – 13) and antitumor activity by certain chemotherapeutic agents (9) have been explained by proposed mechanisms involving an alkylation. The former activity is due to a dialkylphosphorylation of the enzyme.

The monosubstituted amides of dialkoxy(or diphenoxy)thiophosphoric acids were prepared according to a method similar to that reported by Michaelis (14) for the synthesis of dimethylamidodiethoxyphosphate.

The use of triethylamine in N-phosphoryl amino ester synthesis has been reported by Li (15). This tertiary amine was used in the synthesis of the thiophosphoric acid derivatives of DL-methionine and L-proline esters and in the preparation of ethylamidoethoxythionophosphoryl chloride.

Received April 26, 1961, from the College of Pharmacy, University of Illinois, Chicago.

Accepted for publication August 21, 1961.

This paper is abstracted in part from a dissertation submitted to the Graduate College at the Chicago Professional Colleges of the University of Illinois by Florence C. Klee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmaceutical chemistry.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

The authors wish to thank Monsanto Chemical Company for the supply of O,O-diethyl phosphorochloridothioate (Ethyl PCT) used in this investigation.

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EXPERIMENTAL

Thiophosphoryl chloride was prepared by the Knotz method (16) from 24 Gm. (0.75 mole) of powdered sulfur, 100 Gm. (0.73 mole) of phosphorus trichloride, and 2 Gm. (0.015 mole) of aluminum chloride as the catalyst. Reported b.p. 125–126°; yield, 120 Gm. (97.6%). Observed b.p. 124° (uncorr.); yield, 88.5 Gm. (71.8%).

Ethylamidothiophosphoryl chloride, $C_2H_5NHP(S)Cl_2$, was prepared from 32.6 Gm. (0.4 mole) of ethylamine hydrochloride and 136 Gm. (0.8 mole) of thiophosphoryl chloride according to the procedure of Michaelis (14). Reported b.p. 105°/9 mm.; yield is very good. Observed b.p. (uncorr.) 98–102°/8 mm.; yield, 58.0 Gm. (81.5%); n_D^{20} 1.5432.

Anal.—Calcd. for $C_2H_5Cl_2NPS$: N, 7.87; P, 17.40; S, 18.02. Found: N, 7.69; P, 17.49; S, 17.91. Qualitative chemical test for halogen.

Dialkoxyethylamidothiophosphates, $C_2H_5NHP(S)(O\text{-}Alkyl)_2$.—A solution of ethylamidothiophosphoryl chloride (8.90 Gm., 0.05 mole)[1] in anhydrous ether was slowly introduced into a warm sodium alkoxide solution prepared from 2.3 Gm. (0.1 mole) of sodium[2] dissolved in an excess of absolute alcohol. The ester formed upon heating for one-half hour and sodium chloride separated out on cooling. Distilled water was added dropwise to the mixture until the sodium chloride was completely dissolved, and the two layers were transferred to a separator and additional ether was introduced. The ethereal layer was removed, the aqueous layer was extracted again with ether, and the combined ether extracts were dried over anhydrous potassium carbonate. The ether was distilled and the remaining oil was subjected to vacuum distillation. The physical constants, yields, and analyses of the synthesized compounds are shown in Table I.

Diphenoxyethylamidothiophosphate, $C_2H_5N(H)-P(=S)(OC_6H_5)OC_6H_5$.—In 1903, Michaelis (14) reported that the phenyl ester analog of $C_2H_5N(H)-P(=S)(OC_2H_5)OC_2H_5$ is a liquid, but failed to report its method of preparation, analysis, or physical constants. The phenyl ester used in this investigation was prepared in the following manner: sodium phenoxide was prepared from 2.3 Gm. (0.1 mole) sodium in 10 ml. (10.7 Gm., 0.11 mole) of phenol. A small volume of phenol was added to cover the sodium phenoxide. A large excess of phenol was avoided in order to facilitate the isolation of the ester. Ethylamidothiophosphoryl chloride (8.9 Gm., 0.05 mole) in anhydrous ether was added to the cooled sodium phenoxide mixture and warmed on a steam bath for 10 minutes. The cooled mixture was treated with distilled water, extracted, and dried as given in the procedure for the dialkyl esters. After removal of the ether and excess phenol by distillation, the residue decomposed in part when subjected to vacuum distillation, as evidenced by the dark colored residue and evolution of gaseous products. The residue was dissolved in ether and, upon evaporation of the solvent, a crystalline tarry mass resulted. The mass was dissolved in warm 95% ethanol and filtered. Distilled water was added dropwise into the filtrate at 0° until precipitation began to take place. After repeated recrystallization, slow evaporation of the alcoholic solution produced white needle-like crystals rather than the liquid reported by Michaelis. Caution was used in the recrystallization of the product because an oil would form if the proper amount of water was not used. The melting point of the product was 64–64.5° (uncorr.) and the yield was 1.49 Gm. (10.2%).

Anal.—Calcd. for $C_{14}H_{16}NO_2PS$: N, 4.78; P, 10.56; S, 10.93. Found: N, 4.92; P, 10.56; S, 10.68. Quantitative chemical analysis and infrared data are consistent with the desired phenyl ester.

Ethylamidoethoxythionophosphoryl Chloride, $C_2H_5N(H)-P(=S)(OC_2H_5)Cl$.—Ethylthiophosphoryl dichloride ($C_2H_5OP(S)Cl_2$) was prepared according to the method of Bakanova, *et al.* (17), from 17 Gm. (0.1 mole) of thiophosphoryl chloride, 3 Gm. (0.065 mole) of absolute ethanol, and 0.5 Gm. (0.0185 mole) of fine aluminum wire. Reported b.p. 68°/20 mm., yield, 40%. Observed b.p. (uncorr.) 67°/20 mm.; yield, 3.50 Gm. (30%).

A solution of 4.48 Gm. (0.025 mole) of $C_2H_5OP(S)Cl_2$ and 5.06 Gm. (0.05 mole) of triethylamine in 30 ml. of dry chloroform was added dropwise (20 minutes) to a constantly stirred suspension of 2.04 Gm. (0.025 mole) of ethylamine hydrochloride in 25 ml. of dry chloroform at 0°. Stirring was continued at 25° for 40 minutes. A minute quantity of precipitate formed which did not increase in amount even after a 5-day period at 25° followed by heating at 45–50° for 2½ hours. After filtration, the chloroform solution was washed with two 25-ml. portions of distilled water, 1 *N* hydrochloric acid, 10% sodium bicarbonate solution, three 25-ml. portions of distilled water, and then dried over anhydrous sodium sulfate. Removal of the solvent by means of flash evaporation left a residue which distilled as a clear, colorless oil. Observed b.p. (uncorr.) 65–67°/0.25 mm.; n_D^{20} 1.4999; yield, 2.84 Gm. (60.6%).

Anal.—Calcd. for $C_4H_{11}ClNOPS$: N, 7.47; P, 16.51; S, 17.10. Found: N, 7.34; P, 16.59; S, 16.89. Qualitative chemical test for halogen.

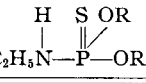
Preparation of Thiophosphoric Acid Derivatives of DL-Methionine Ethyl Ester.

Synthesis of the Ethyl Ester of DL-Methionine.—It was found that the highest yield of ester was obtained when a suspension of 14.92 Gm. (0.1 mole) DL-methionine in 150 ml. of absolute ethanol was saturated at 0° with hydrogen chloride and refluxed for 1 hour. After a 48-hour period, most of the solvent was removed by use of a flash evaporator. The remaining viscous yellow liquid was dissolved in 40 ml. of absolute ethanol, 50 ml. of anhy-

[1] The ethyl ester was prepared from 0.2 mole (35.6 Gm.) and the isobutyl ester from 0.08 mole (14.2 Gm.) of ethylamidothiophosphoryl chloride.

[2] Potassium was used to make the alkoxide of tertiary butyl alcohol since the reaction was too sluggish with sodium and the yield was less than that obtained with the more active metal.

TABLE I.—DIALKOXYETHYLAMIDOTHIOPHOSPHATES



R	B.p.,[a] °C.	n_D^{20}	Yield, Gm.	Yield, %	Analyses, % Calcd.[b] N	Calcd. P	Calcd. S	Found N	Found P	Found S
C_2H_5[c]	67–70	1.4716	26.6	67.4	7.10	15.71	16.26	6.99	15.71	16.21
n-C_3H_7	84–87	1.4695	7.9	70.1	6.22	13.75	14.23	6.06	13.84	14.00
iso-C_3H_7	72–75	1.4617	8.3	73.7	6.22	13.75	14.23	6.10	13.92	14.14
n-C_4H_9	112–116	1.4690	9.0	70.6	5.53	12.23	12.66	5.47	12.14	12.48
sec-C_4H_9	87–90	1.4658	5.8	45.8	5.53	12.23	12.66	5.68	12.14	12.65
tert-C_4H_9	78–81	1.4662	4.9	38.7	5.53	12.23	12.66	5.48	12.04	12.47
iso-C_4H_9	90–92	1.4640	14.6	71.8	5.53	12.23	12.66	5.38	11.95	12.51

[a] Uncorrected b.p. at 0.25 mm. [b] Calculated for $C_6H_{16}NO_2PS$ (ethyl ester); $C_8H_{20}NO_2PS$ (propyl esters); $C_{10}H_{24}NO_2PS$ (butyl esters). [c] Reported in literature (14), b.p. 94°/12 mm.; yield, none reported.

drous ether was added, and anhydrous ammonia gas was bubbled through the two phase system at 0° for 1 hour. After 66 hours, the ammonium chloride and any unreacted amino acid were removed by filtration. The solvent was removed by flash evaporation and the residue subjected to vacuum distillation, giving 12.5 Gm. (70.3%) of the ethyl ester as a clear, colorless, oil. Observed b.p. (uncorr.) 84–86°/0.25 mm. Reported (18) b.p. 112°/2.5 mm.; yield, 88%.

Synthesis of N-(Diethoxyphosphinothioyl)-DL-methionine Ethyl Ester.—A dry chloroform solution (25 ml.) of O,O-diethyl phosphorochloridothioate (Ethyl PCT-Monsanto) (2.36 Gm., 0.0125 mole) was added dropwise within 60 minutes to a constantly stirred dry chloroform solution (25 ml.) of DL-methionine ethyl ester (2.19 Gm., 0.0124 mole) and triethylamine (1.25 Gm., 0.0125 mole) at 0–5°. Stirring was continued for 30 minutes at room temperature and after 18 hours the chloroform solution was washed and dried in the same manner as ethylamidoethoxythionophosphoryl chloride. A flash evaporator was used to remove chloroform and any remaining halogen-containing reactant. The ester distilled as a clear, colorless oil. Observed b.p. (uncorr.) 145–147°/0.25 mm.; n_D^{20} 1.4950; yield, 2.31 Gm. (56.8%).

Anal.—Calcd. for $C_{11}H_{24}NO_4PS_2$: N, 4.25; P, 9.40; S, 19.47. Found: N, 4.23; P, 9.47; S, 19.57.

Synthesis of N-(Ethoxychlorophosphinothioyl)-DL-methionine Ethyl Ester.—This ester was prepared according to the directions given for N-(diethoxyphosphinothioyl)-DL-methionine ethyl ester. The ethylthiophosphoryl dichloride (4.48 Gm., 0.025 mole) was added within 70 minutes to 4.43 Gm. (0.025 mole) of DL-methionine ethyl ester and 2.53 Gm. (0.025 mole) of triethylamine. This ester, a clear yellow oil, underwent complete decomposition when high vacuum distillation was attempted. n_D^{20} 1.4962; yield, 6.18 Gm. (77.3%).

Anal.—Calcd. for $C_9H_{19}ClNO_3PS_2$: N, 4.38; P, 9.69; S, 20.06. Found: N, 4.22; P, 9.65; S, 19.83. Qualitative chemical test for halogen.

Synthesis of N-(Dichlorophosphinothioyl)-DL-methionine Ethyl Ester.—This derivative was prepared in the same manner as the N-(diethoxyphosphinothioyl)-DL-methionine ethyl ester. Thiophosphoryl chloride (2.99 Gm., 0.0176 mole) was added within 180 minutes to 3.13 Gm. (0.0176 mole) of DL-methionine ethyl ester and 1.79 Gm. (0.0176 mole) of triethylamine. The product was washed with four 25-ml. portions of each solution instead of the indicated number. This derivative, a clear yellow oil, underwent complete decomposition when high vacuum distillation was attempted. Yield, 3.76 Gm. (68.6%). It was observed that the dichloro derivative was unstable at room temperature, since approximately 10 days after its synthesis, the yellow oil started to decompose.

Preparation of the Thiophosphoric Acid Derivatives of L-Proline Ethyl Ester

Synthesis of the Ethyl Ester of L-Proline.—It was found that the following procedure gives a 65% yield of the ester from 0.109 mole of L-proline. Dry hydrogen chloride gas was introduced very slowly (4 hours) into a constantly stirred suspension of L-proline (12.58 Gm., 0.109 mole) in absolute ethanol (150 ml.) at 0°. After 2 days at 25°, the solvent was removed by flash evaporation and the residue dissolved in absolute ethanol (40 ml.). After the addition of anhydrous ether (50 ml.), dry ammonia gas was introduced slowly at 0° until saturation occurred (1 hour). After an 18-hour period the ammonium chloride was removed by filtration and washed with anhydrous ether. The ether of the combined ethereal solutions was removed by flash evaporation and the residue was subjected to vacuum distillation. Reported (19) b.p. 78°/12–14 mm.; yield, 40.5 Gm. (66%). Observed b.p. (uncorr.) 77–78°/12 mm.; yield, 10.1 Gm. (64.5%).

Synthesis of N-(Diethoxyphosphinothioyl)-L-proline Ethyl Ester.—A dry chloroform solution (25 ml.) of O,O-diethyl phosphorochloridothioate (4.72 Gm., 0.025 mole) (Ethyl PCT-Monsanto) was introduced within 30 minutes into a constantly stirred dry chloroform solution (25 ml.) of proline ethyl ester (3.58 Gm., 0.025 mole) and triethylamine (2.53 Gm., 0.025 mole) at 0°. Stirring was continued for 30 minutes and the solution was allowed to stand at 25° for 18 hours.[3] The chloroform solution was washed, dried, and distilled in the same manner as the ethylamidoethoxythionophosphoryl chloride. This ester is a clear, colorless, distillable oil. Observed b.p. (uncorr.) 118–120°/0.25 mm.; n_D^{20} 1.4810; yield, 4.65 Gm. (63.0%).

[3] This derivative was heated for 1 hour at 55–60° on 2 successive days to insure completeness of reaction. It was found that the quantity of white precipitate that had originally formed did not increase in amount upon further heating.

Anal.—Calcd. for $C_{11}H_{22}NO_4PS$: N, 4.74; P, 10.49; S, 10.86. Found: N, 4.68; P, 10.47; S, 10.86.

Synthesis of N-(Ethoxychlorophosphinothioyl)-L-proline Ethyl Ester.—This ester was prepared in a similar manner as the N-(diethoxyphosphinothioyl)-L-proline ethyl ester. Ethylthiophosphoryl dichloride (3.97 Gm., 0.022 mole) was introduced within 120 minutes into the proline ethyl ester (3.18 Gm., 0.022 mole) and triethylamine (2.24 Gm., 0.022 mole). No additional heating was applied after the solution was allowed to stand at 25° for 18 hours. The ester is a clear, yellow, undistillable oil. n_D^{25} 1.5088; yield, 4.81 Gm. (75.9%).

Anal.—Calcd. for $C_9H_{17}ClNO_3PS$: N, 4.90; P, 10.84; S, 11.23. Found: N, 4.83; P, 10.86; S, 10.99. Qualitative chemical test for halogen.

Synthesis of N-(Dichlorophosphinothioyl)-L-proline Ethyl Ester.—This derivative was prepared according to the procedure given for the N-(diethoxyphosphinothioyl)-L-proline ethyl ester. Thiophosphoryl chloride (4.24 Gm., 0.025 mole) was introduced within 120 minutes into the proline ethyl ester (3.58 Gm., 0.025 mole) and triethylamine (2.53 Gm., 0.025 mole). No additional heating was applied after the solution was allowed to stand at 25° for 18 hours. The ester is a clear, yellow, undistillable oil. Yield, 5.67 Gm. (82.2%). As was found in the methionine series, this dichloro derivative is not stable at room temperature.

Colorimetric Determination of Phosphorus.—The thiophosphoric acid derivatives of ethylamine, methionine, and proline were analyzed for phosphorus by the method of King (20). The following modifications were introduced: A standard curve was made up from aliquots (containing 0.01 to 0.15 mg. of phosphorus) of a standard phosphate solution. Readings were obtained in a Coleman spectrophotometer at 650 mμ. King compares the color of a standard phosphate solution with that of the unknown solution in a Duboscq colorimeter.

Double quantities of 72% perchloric acid, 5% ammonium molybdate, and sulfonic acid were used and diluted to 100 ml. instead of 15 ml.

The sample (approximately 40 mg.) was digested with 1 ml. of perchloric acid,[4] diluted to 100 ml. with distilled water and an aliquot (usually 1 ml.), containing between 0.05 and 0.1 mg. phosphorus, was used for the colorimetric determination. Results were very satisfactory.

DISCUSSION

Since the yield of DL-methionine ethyl ester when prepared by the method of Booth, *et al.* (18), did not furnish enough starting material for further syntheses, the method of preparation was investigated to improve the yield. The highest yield of ester was obtained by the procedure given in the experimental section.

Kapfhammer and Matthes (19) reported the synthesis of the ethyl ester of L-proline from 50 Gm. of L-proline with no mention of the amount of ethanol, rate of hydrogen chloride introduction, time, and temperature at which the reaction took place. It was found in this investigation that a 65% yield of the ester could be obtained from 12.58 Gm. of L-proline according to the directions given in the experimental part. Freshly prepared ester was used in the syntheses of the thiophosphoramides, since this ester loses ethanol spontaneously at room temperature, to form the solid anhydride or diketopiperazine derivative.

The thiophosphoric acid derivatives of both amino acid ethyl esters were prepared from the amino acid ester and an appropriate thiophosphoryl chloride (1:1 molar ratio) in the presence of triethylamine. The tertiary amine reacted with the liberated hydrogen chloride gas and allowed most of the amino acid ester to form the desired product instead of the amino acid ester hydrochloride.

Analytical Methods.—Nitrogen analyses were by a semimicro Kjeldahl method and phosphorus analyses were determined by a modified King method. With the exception of the methionine series compounds, sulfur was determined gravimetrically after a perchloric-nitric acid oxidation method of Webster and Powers (21). Callan and Toennies (22) have reported the incomplete oxidation of sulfur in methionine when wet-oxidative methods were used. In this investigation it was found that the methionine derivatives were incompletely oxidized by perchloric-nitric acid, but completely oxidized when they were burned in the Parr bomb. The sulfur was then determined gravimetrically as barium sulfate. Halogen was detected by a qualitative chemical test after a sodium fusion of the halogen-containing compounds in the three series.

SUMMARY

The thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters of the general formula, $-\overset{|}{N}-\overset{\overset{S}{\|}}{P}(X)_2$ where $(X)_2$ is $(O\text{-alkyl})_2$, $(O\text{-phenyl})_2$, $(C_2H_5O)Cl$, or $(Cl)_2$, were synthesized for the investigation of their possible enzyme inhibition *in vivo*.

It was found that the phenyl ester is crystalline rather than liquid as reported by Michaelis who failed to report its method of preparation, analysis, and physical constants. The procedures for obtaining good yields of the ethyl esters of these two amino acids from 0.1 mole of the amino acid are given.

All of the compounds, with the exception of the methionine derivatives, were oxidized by the wet-oxidative perchloric-nitric acid method of Webster and Powers. The methionine derivatives were completely oxidized in the Parr bomb.

It was found that a modified King method gave very satisfactory results for the colorimetric determination of phosphorus.

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[4] In some cases where oxidation was slow, a drop or two of nitric acid was added. Heating was continued for 3 or 4 minutes after the mixture had been colorless to drive off the excess of nitric acid.

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Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters II

Biological Activities in Guppies

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Observed biological responses of guppies in 0.01% w/v aqueous solutions of the thiophosphoric acid derivatives indicate that the compounds are acetylcholinesterase inhibitors. The 0.001% solutions furnished additional information concerning the biological activity of these compounds. Structure-toxicity relationship is discussed.

THE SYNTHESIS of thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters has recently been published (1).

The thiophosphoric acid derivatives used in this investigation have the general formula

—N—P(X)$_2$, where (X)$_2$ = (O-alkyl)$_2$, (O-C$_6$H$_5$)$_2$, (C$_2$H$_5$O)Cl, or (Cl)$_2$, and N represents the amine or amino acid ester moiety. These structures are similar to those of known anticholinesterases, thus the thiophosphoric acid derivatives may be acetylcholinesterase inhibitors. If so, then the following types of biological responses may be visualized in guppies (*Lebistes Reticulatus*): difficulty in breathing, dizziness, evacuation of rectum and bladder, convulsions, and total paralysis (death).

The purpose of this investigation was to observe the biological responses of baby guppies which have been immersed for 24 hours in 0.01 and 0.001% w/v aqueous solutions of each compound.

EXPERIMENTAL

Table I lists the compounds used in this study. Stock aqueous solutions or aqueous suspensions (0.1% w/v) were prepared from which 0.01 and 0.001% w/v solutions were obtained after making a tenfold dilution of the stock and 0.01% w/v solution.

An alcoholic stock solution and alcoholic dilutions were made of diphenoxyethylamidothiophosphate, since it was not possible to obtain a uniform 0.1% w/v aqueous suspension. Aliquots of the alcoholic solutions were allowed to evaporate to dryness and a volume of distilled water equal to the volume of alcohol was introduced before testing the biological activity of the compound.

Ten milliliters of 0.01 and 0.001% w/v aqueous solutions of each compound were placed in separate vials (2.4 cm. diameter and 5.5 cm. in height) and 10 young guppies were placed in each vial. For control purposes, distilled water was used instead of an aqueous solution of a compound under test and it was observed that all of the guppies survived after 24 hours. The O,O-diethyl phosphorochloridothioate (Ethyl PCT-Monsanto), C$_2$H$_5$O—P(S)—Cl,

Received April 28, 1961, from the College of Pharmacy, University of Illinois, Chicago.
Accepted for publication August 21, 1961.
This paper is abstracted in part from a dissertation submitted to the Graduate College at the Chicago Professional Colleges of the University of Illinois by Florence C. Klee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmaceutical chemistry.
Presented to the Scientific Section, A. PH. A., Chicago meeting, April 1961.
The authors are grateful to Dr. Katsuyuki Yokoyama for his generous supply of guppies and helpful suggestions.
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TABLE I.—PER CENT EXPIRED OF GUPPIES

No.	Compound	Moles/L. × 10^{-4} 0.01% w/v	% Expired 24 hr., 0.01% w/v	% Expired 24 hr., 0.001% w/v
1	$C_2H_5NHP(S)(OC_2H_5)_2$	5.07	100	0
2	$C_2H_5NHP(S)(OCH_2CH_2CH_3)_2$	4.44	100	10
3	$C_2H_5NHP(S)(OCH(CH_3)_2)_2$	4.44	100	0
4	$C_2H_5NHP(S)(OCH_2CH_2CH_2CH_3)_2$	3.95	100	40
5	$C_2H_5NHP(S)(OCH(CH_3)CH_2CH_3)_2$	3.95	100	0
6	$C_2H_5NHP(S)(OC(CH_3)_3)_2$	3.95	100	20
7	$C_2H_5NHP(S)(OCH_2CH(CH_3)_2)_2$	3.95	100	20
8	$C_2H_5NHP(S)(OC_6H_5)_2$	3.41	100	100
9	$C_2H_5NHP(S)(OC_2H_5)Cl$	5.33	100	10
10	$C_2H_5NHP(S)Cl_2$	5.62	100	90
11	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)(OC_2H_5)_2$	3.04	100	20
12	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)(OC_2H_5)Cl$	3.13	100	0
13	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)Cl_2$	3.22	100	0
14	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)(OC_2H_5)_2$	3.39	100	50
15	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)(OC_2H_5)Cl$	3.50	70	0
16	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)Cl_2$	3.62	100	0
17	$(C_2H_5O)_2P(S)Cl$ Reference standard	5.31	100	0

an anticholinesterase (2), was chosen as the reference standard for making biological response comparisons.

The behavior of the fish was closely observed for 2 hours, and at the end of 24 hours the number of fish which survived was recorded. There were no survivors in the 0.01% w/v aqueous solutions of the compounds with the exception of N-ethoxychlorophosphinothioyl-L-proline ethyl ester (Table I). The three fish (survivors) matured in 1 month and the male and a female expired shortly thereafter.

The 0.01% w/v (3.04 to 5.62 × 10^{-4} *M*) aqueous solutions of the compounds produced biological responses similar to those of known acetylcholinesterase inhibitors. Before total paralysis occurred, the guppies exhibited difficulty in breathing and intermittent convulsions (head and tail flutter) followed by coma. Many of the fish lost their sense of balance and were seen in abnormal positions.

An unusual spinal effect was seen when the fish were subjected to the diphenoxyethylamidothiophosphate (0.01% w/v). This compound caused the bending of the tail of several fish to such an extent that the tip of the caudal fin was adjacent to the head of the fish.

The fish which survived after 24-hour chemical treatment with aqueous 0.001% w/v solutions were transferred to fresh water and observed periodically for several months to determine the sex, the months required for the fish to mature, and any abnormal development.

Behavior Toward Food.—An interesting observation concerning the behavior of the fish in fresh water toward food was made immediately after the 24-hour chemical treatment with 0.001% solutions of the compounds (Table II). Most of the fish consumed the food, but the fish which had been in solutions of the dihalo and four carbon chain derivatives, ignored the food. Fish previously in contact with the *sec*-butyl ester, actually leaped out of the water in an attempt to get away from the food which had touched them.

Sex Differentiation(?)—Unfortunately, the female/male ratio cannot be established at the start of the experiment since it is impossible to determine the sex of a baby guppy. However, after the guppies matured, it was found that the female/male survival ratio was greater than two to one, since there were more female survivors than males when both sexes survived (Table II). The larger number of female survivors may be due to the possibility that the compounds are more toxic to the male guppies or else most of the baby guppies (sex unknown) are females. The first reason seems more probable, since experimentation involving mature fruit flies shows that in general, the compounds are more toxic to the males than to females.

It is interesting to note that with the exception of the ethoxychloro derivative in the proline series, there were no male survivors in the ethyl and ethoxychloro derivatives in the three series of compounds, the reference standard, the normal, and isobutyl esters.

It is noteworthy that only the male fish survived in the aqueous solution of the normal propyl ester.

Effect on Growth.—Normal guppies mature in about 3 months after birth as evidenced by full

TABLE II.—SOME BIOLOGICAL RESPONSES OF GUPPIES

Compound[a]	% Expired 24 hr.	Behavior[b] Toward Food	Sex Survived[c] and Growth[d]
$X(OC_6H_5)_2$	100	..	None
XCl_2	90	B	None[e]
$X(OCH_2CH_2CH_2CH_3)_2$	40	B	F(n)
$X(OC(CH_3)_3)_2$	20	B	M(n) F(n)[f]
$X(OCH_2CH(CH_3)_2)_2$	20	B	F(n)
$X(OC_2H_5)Cl$	10	A	F(R)
$X(OCH_2CH_2CH_3)_2$	10	A	M(n)
$X(OCH(CH_3)_2)_2$	0	A	M(n) F(n)
$X(OCH(CH_3)CH_2CH_3)_2$	0	C	M(n) F(n)
$X(OCH_2CH_3)_2$	0	A	F(n)
$Y(OCH_2CH_3)_2$	20	A	F(n)
$Y(OC_2H_5)Cl$	0	A	F(R) + I_2
YCl_2	0	A	M(R) F(R)
$Z(OCH_2CH_3)_2$	50	A	F(R)
$Z(OC_2H_5)Cl$	0	A	M(R) F(R)
ZCl_2	0	A	M(R) F(R)
$(C_2H_5O)_2P(S)Cl$	0	A	F(R) + I_1

[a] The compounds in each series are arranged according to decreasing toxicity as determined by the per cent expired after 24 hours in the 0.001% w/v aqueous solutions. $(C_2H_5O)_2P(S)Cl$ is the reference standard; X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N{-}P(S)$. [b] A, Consumed food; B, ignored food; C, darted away from food. [c] M, Male; F, female. [d] n, Normal, fish matured in 3 months; R, rapid, fish matured in two months; I_1 inhibited or retarded, fish were immature after 3 months; I_2, inhibited or retarded, fish were immature after 6 months. [e] The remaining fish expired 3 hours after transfer to fresh water. [f] Gave birth to young.

development of the body. Caudal fin pigmentation and modification of the pelvic fins in the male as claspers are secondary sexual characteristics which can be easily observed.

Growth Stimulation.—It was noted that both male and female fish matured in 2 months when they had been in aqueous solutions of the dihalo derivatives in the methionine and proline series, and the ethoxychloro derivative in the latter series (Table II). Female guppies, the only survivors, matured in 2 months after being in contact with aqueous solutions of the ethoxychloro derivatives in the ethylamine and methionine series, reference standard, and the ethyl derivative in the proline series.

The greatest stimulation of growth was produced by the aqueous solution of N-(dichlorophosphinothioyl)-L-proline ethyl ester. Gigantism, or abnormal overgrowth, of the female and male fish, which are slightly more than twice the size of normal fish of the same age, was observed.

Growth Inhibition or Retardation.—After 3 months there is no apparent change in size of some of the fish which survived in aqueous solutions of the ethoxychloro derivative in the methionine series and reference standard. The fish which had been exposed to the methionine derivative are the same size and immature even at 6 months of age (Table II). It is possible that these fish are males, since the only fish which did mature are females.

Abnormal Body Development.—The female fish which survived in the aqueous solution of the ethoxychloro ester (methionine series) do not have well-proportioned bodies. Their short bodies and unusually long caudal fins give the fish a "stretched out" appearance. The ventral side is not curved like that of normal fish, but appears to be rather straight. This peculiar shape was noted in other female fish but, unfortunately, the previous chemical treatment is unknown since all the female survivors were placed into the same vessel with all the male survivors for the purpose of observing whether reproduction can occur. The female fish which had matured rapidly and were not well-proportioned expired at the age of $4^1/_2$ months.

In contrast, the giant female fish resulting from the 24-hour contact with the dichloro derivative in the proline series are beautifully proportioned and appear to be in perfect health at 6 months of age.

Reproduction.—The guppies which had undergone 24-hour contact with the same compound were placed into 2 L. of fresh water and observed periodically until they matured. Only the fish which had been in the aqueous solution of the tertiary butyl ester produced progeny (Table II).

The male and female survivors were introduced into the same container and after 6 months, it is interesting to note, only one of the many females had given birth to young. There is a possibility that the synthetic thiophosphoric acid derivatives cause sterility in guppies in view of the fact that alkylating agents are known to produce transient sterility in male rats (3).

Tail Pigment.—Normal female guppies do not have highly pigmented tails. The tip of the caudal fin is a very pale yellow and practically unnoticeable. The giant female fish which resulted after 24-hour contact with an aqueous solution of the N-(dichlorophosphinothioyl)-L-proline ethyl ester have large tails and the bright yellow pigment near the end of the fin can be easily seen in daylight. The ends of the caudal fins of the females which had been exposed to other compounds are green and black.

The bodies and tail of normal male guppies are orange, green, blue, and black. The pigment of the male survivors is essentially the same as that of fish which have not been in 24-hour contact with thiophosphoric acid derivatives, however, there appears to be a predominance of brilliant red color and almost complete absence of orange pigment.

DISCUSSION

The structures of the synthesized compounds are similar to those of compounds known to be acetylcholinesterase inhibitors. Since very frequently the anticholinesterase activity *in vitro* of a given organophosphorus compound is not related to its toxicity *in vivo*, guppies were chosen to investigate the biological activity of the thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters. Fish possess acetylcholinesterase in addition to other enzymes, and their biological responses can be readily observed when enzyme inhibition occurs.

If these derivatives are acetylcholinesterase inhibitors then certain biological responses such as difficulty in breathing, dizziness, and convulsions should have been observed. These responses were observed but other responses such as growth stimulation and retardation were also noted. It appears to the authors that the compounds probably have anticholinesterase activity.

The compounds of the three series are arranged in Table III according to decreasing toxicity as determined by the expiration time of the fish which represents time, in minutes, when the last fish of each group of 10 ceased to breath. For making biological response comparisons, $(C_2H_5O)_2P(S)Cl$, an anticholinesterase, was chosen as the reference standard.

Structure-Toxicity Relationship.—The compounds as 0.01% solutions are toxic to guppies. Based on the maximum time for expiration (Table III), in general, the ethylamine derivatives are faster acting than the methionine derivatives, and the latter are faster acting than the proline derivatives. As the expiration time decreases, most probably the toxicity increases, and with this assumption, the following observations concerning toxicity of the compounds are made.

Varying X[1] Groups of the Amine[2]—P—Nucleus:



Ethylamine Series, $C_2H_5N—P(X)_2$.—Observations of the results obtained with the 0.01% solutions indicate that halogen derivatives are the most toxic to fish (Table III). Branching of the three carbon chain produces an ester which is one-third as toxic as the straight chain ester because the expiration time is almost tripled. The four carbon esters are about one-half as toxic as the normal propyl ester. The two carbon ester is the least toxic since the biological responses of the guppies were observed for almost 2 hours.

Comparison of the results observed with the 0.001% solutions indicate that the dichloro and phenyl esters are the most toxic to guppies because there were no survivors (Table II). Both sexes survived in the isopropyl, secondary, and tertiary butyl esters; however, it is interesting to note that there were more female survivors than males. The fish which had been exposed to the tertiary butyl ester produced offspring. Only females survived in the normal butyl, isobutyl, ethyl, and ethoxychloro esters. The latter ester stimulated growth since the females matured in 2 months. There were exclusively male survivors in the normal propyl ester which suggests that females are more susceptible than males to this compound.

Varying X[3] Groups of the Amine[2]—P—Nucleus: DL-

Methionine Series, DL-$CH_3SCH_2CH_2CH$—NH—P

$(X)_2$; **L-Proline Series,** L-$CH_2CH_2CH_2CHN$—$P(X)_2$

—In the two amino acid series, the 0.01% solutions of the diethyl esters are the most toxic to guppies. The diethoxy and dichloro derivatives are more toxic (quicker acting) than the ethoxychloro ester (Table III). However, the proline derivatives are less toxic (slower acting) than the corresponding methionine derivatives. The ethoxychloro ester in the proline series is the least toxic of the amino acid derivatives because there are survivors.

The 0.001% solutions furnished additional information in regards to the biological activity of these compounds in guppies.

When the X groups attached to the methionine nucleus[4] are ethoxy and chlorine, the ester has the unusual ability to retard or inhibit the growth of some of the guppies (possibly males) and stimulated the growth of the females, since the only fish which matured are females (Table II). Replacement of the ethoxy group by chlorine produced the dihalo derivative which caused rapid growth of both females and males. The latter matured in only 2 months. When X groups are OC_2H_5, only females survived which matured in about 3 months.

As 0.001% solutions, the proline derivatives obtained when diethoxy, ethoxychloro, and dichloro groups are attached to the proline nucleus[5] produced rapid growth in female guppies. The latter two compounds also caused rapid growth in the males that survived. The dichloro derivative stimulated growth to such an extent that both male and female fish are more than twice the size of normal fish of the same age.

Varying the Amine[2] of the —P—OC_2H_5 **Nucleus.**



—It was found that the methionine derivative is more toxic than the ethylamine and proline derivatives (Table III). Female guppies are the only survivors in the 0.001% solutions of these compounds (Table II). The proline derivative stimulated growth as evidenced by the females maturing in a relatively short time.

Varying the Amine[2] of the —P—OC_2H_5 **Nucleus.**



—The ethylamine derivative is much more toxic than the methionine derivative which, in turn, is more potent than the proline derivative (Table III). All of these esters (0.001%) promoted rapid growth of the females and the proline ester, in addi-

[1] $(X)_2$ = $(OC_2H_5)_2$, $(OC_2H_5)Cl$, and $(Cl)_2$.

[2] Ethylamine or the DL-methionine and L-proline ethyl esters.

[4] DL-Methionine nucleus, DL-$CH_3SCH_2CH_2CH$—NH—P—

[5] L-Proline nucleus, L-$CH_2CH_2CH_2CH$—N—P—

TABLE III.—EXPIRATION TIME OF GUPPIES IN 0.01% w/v AQUEOUS SOLUTION OF THE COMPOUND[a]

Minutes	Compound	Minutes	Compound
	Ethylamine Series[b]		DL-Methionine Series[c]
12	$X(OC_2H_5)Cl$	16	$Y(OC_2H_5)_2$
	XCl_2	30	YCl_2
20	$X(OCH_2CH_2CH_3)_2$	257	$Y(OC_2H_5)Cl$
	$X(OC_6H_5)_2$		L-Proline Series[d]
37	$X(OCH_2CH(CH_3)_2)_2$	120	$Z(OC_2H_5)_2$
	$X(OCH_2CH_2CH_2CH_3)_2$	180	ZCl_2
46	$X(OCH(CH_3)CH_2CH_3)_2$	1440	$Z(OC_2H_5)Cl$[e]
	$X(OC(CH_3)_3)_2$		
56	$X(OCH(CH_3)_2)_2$		
112	$X(OC_2H_5)_2$		

[a] Reference standard, $(C_2H_5O)_2P(S)Cl$, expiration time, 11 minutes; for control purposes, distilled water was used instead of the 0.01% solution of the compound under test, and it was observed that all of the guppies were alive after 24 hours. [b] X = $C_2H_5NHP(S)$. [c] Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$. [d] Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)$. [e] Survival of 30% after 24 hours.

tion, had the same effect on males (Table II). The methionine ester is outstanding in that it retarded or inhibited the growth of some of the guppies (possibly the males).

Varying the Amine[2] of the —P(S)Cl Nucleus.—



The order of decreasing toxicity is ethylamine, methionine, followed by proline (Table III). There were no survivors in the 0.001% solution of the ethylamine derivative (Table II). The amino acid derivatives actually stimulated the growth of both sexes. The proline derivative excelled in this ability in that it caused gigantism.

SUMMARY AND CONCLUSIONS

The observed biological responses of baby guppies which had been immersed in 0.01% w/v aqueous solutions of the thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters, indicate that these derivatives probably have anticholinesterase activity. It was noted that the phenyl ester caused extreme bending of the caudal fin of some of the fish.

Other biological responses were observed with the 0.001% solutions. There were very few male survivors, and only one of the many female survivors produced progeny. Behavior toward food, growth stimulation, growth retardation or inhibition, abnormal body development, and enhancement of pigmentation were observed.

Almost all of the amino acid derivatives, the ethoxychloro derivative (ethylamine series), and the reference standard caused rapid growth of both sexes or of the female survivors. Maximum growth stimulation was observed with the dichloro proline derivative. The female fish which had matured rapidly and were not well-proportioned expired at the age of $4\frac{1}{2}$ months.

The ethoxychloro methionine derivative and the reference standard caused growth inhibition of some of the guppies (possibly the males) and stimulated the growth of the females.

There were exclusively male survivors in the normal propyl ester.

The fish which had been in the dichloro and butyl derivatives (ethylamine series) ignored food. The guppies previously in contact with the *sec*-butyl ester actually leaped away from the food which had touched them.

Pigmentation was enhanced in both sexes.

Based on the maximum time for expiration, in general, the ethylamine derivatives are the fastest and the proline derivatives are the slowest acting. Structure-toxicity relationship is discussed. Observations of the results obtained with the 0.01% solutions indicate that halogen derivatives (ethylamine series) and diethoxy derivatives (both amino acid series) are the most toxic to guppies.

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Loss of Sensitivity in Distinguishing Real Differences in Dissolution Rates Due to Increasing Intensity of Agitation

By W. E. HAMLIN, E. NELSON†, B. E. BALLARD†, and J. G. WAGNER

Two polymorphic forms of methylprednisolone, designated I and II, were compressed into constant surface pellets and their dissolution rates determined *in vitro* by four different methods and *in vivo* following implantation in rats. There was a highly significant difference between the rates of dissolution of forms I and II *in vivo*. There were also highly significant differences between the rates of dissolution of forms I and II in the two *in vitro* tests where the intensity of agitation was of a relatively low order of magnitude. There were no significant differences in the rates of dissolution of forms I and II in the two *in vitro* tests which produce the greatest velocity of dissolution medium across the surface of the pellet. One of the *in vitro* methods which gave no significant difference in rates for forms I and II employed the machine of Souder and Ellenbogen, rotating at 40 r.p.m. This machine is now being used in interlaboratory tests of timed release and prolonged action oral products in an attempt to "develop a test procedure which may be generally useful as a routine control procedure." The implications of our results in relation to such testing procedures are discussed.

IN PERFORMING dissolution rate experiments several investigators (1–6) have used relatively high intensities of agitation, and Nelson (7) has described a method in which free convection, diffusion coefficient, and concentration were stated to be the rate determining factors. Several authors (4, 5, 8) have reviewed the literature concerning the effect of agitation speed on the velocity of heterogeneous reactions and the kinetics of dissolution; some of these results are discussed in the Appendix. We have studied the *in vitro* dissolution of many substances by several methods and have attempted in some cases to correlate the results with *in vivo* measurements of absorption rate, either after implantation of the substances or following their oral administration. With respect to relative intensity of agitation (rate of stirring or velocity of the dissolution medium across the surface of the dissolving substance), we have been interested in determining: (*a*) what *in vitro* method correlates best with *in vivo* results; (*b*) whether differences between substances or lots of the same substance or product can be differentiated with respect to dissolution rate at different intensities of agitation; and (*c*) whether the error in the measured dissolution rate is independent of or dependent upon the relative intensity of agitation. Results of such investigations are important, not only from a theoretical viewpoint but also from a very practical viewpoint. Recently the Pharmaceutical Contact Section of the Pharmaceutical Manufacturer's Association (9) has been carrying out interlaboratory tests on timed release and prolonged action oral products. These investigations are intended to "develop a test procedure which may be generally useful as a routine control method." One of us (J. G. Wagner) wrote a memorandum (10) to the Tablet Subcommittee of the Contact Section outlining some of the pitfalls in the current tests under consideration. One of the pitfalls mentioned was that the intensity of agitation resulting from use of the machine described by Souder and Ellenbogen (11), and used in these interlaboratory tests, may be too great. On the basis of theory it was hypothesized that there may be examples where products would give a significant difference in results *in vivo*, yet by such a method the difference would not be measurable *in vitro*. We now have some experimental data obtained *in vivo* and by several *in vitro* methods to support this viewpoint.

EXPERIMENTAL

In order to test the above hypothesis we chose to use constant surface pellets of two polymorphic forms of methylprednisolone, hereafter termed forms I and II. Form II has a greater water solubility than form I, hence one could deduce that form II would have a greater initial dissolution rate in water than form I on the basis of dissolution rate theory (8). We chose to use pellets compressed at high pressures since with methylprednisolone I and II we observed these remained intact during dissolution and the surface areas during the experiments were accurately known. By operating in the region where the concentration of methylpredni-

Received August 7, 1961, from the Pharmacy Research Section, Product Research and Development Unit, The Upjohn Co., Kalamazoo, Mich.

Accepted for publication August 21, 1961.

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Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

The authors greatly appreciate the samples of the polymorphic forms of methylprednisolone supplied by Dr. John W. Shell, The Upjohn Co.

solone in the dissolution medium was very small compared with the concentration of a saturated solution, the mathematics was greatly simplified. In the *in vitro* tests such low concentrations were readily determined by means of ultraviolet spectrophotometry using the Cary recording spectrophotometer. See Fig. 1. In the implantation experiments in rats, rate of loss of weight was used as a criterion of dissolution and absorption rate. However, for constant surface pellets we have correlated rate of loss of weight with rate of appearance in the dissolution medium. Most of the details of the *in vivo* and *in vitro* experiments are given below. An adequate description of the pellet holder and the Wruble machine (12) will be made at a later date.

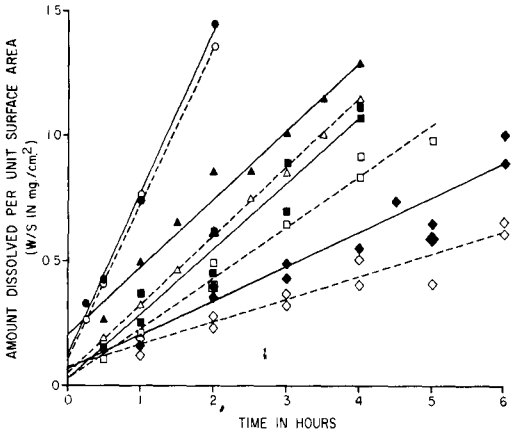


Fig. 1.—Pseudo zero-order plots for dissolution of methylprednisolone polymorphs I and II from constant surface pellets in water at 37°. ●—Polymorph II and ○—polymorph I, pellet holder method, 40 r.p.m. in the Souder and Ellenbogen machine. ▲—Polymorph II and △—polymorph I, pellet holder method, 12 r.p.m. in the Wruble machine. ■—Polymorph II and □—polymorph I, pellet holder method, 6 r.p.m. in the Wruble machine. ◆—Polymorph II and ◇—polymorph I, hanging pellet method.

Pellet Implants in Rats.—Pellets of pure methylprednisolone I and methylprednisolone II, 0.25 inch in diameter, were compressed at high pressure. Two disks of one polymorph were implanted in each rat. After different intervals of time the pellets were removed and weighed. Knowing the loss in weight, the surface area, and the time of exposure, the rate of weight loss, equivalent to the rate of absorption with opposite sign, was calculated. Thirty separate determinations involving three series of 10 different rats were used with methylprednisolone I, and 15 separate determinations involving three series of five different rats were used with methylprednisolone II. The overall average rates obtained are shown in Table I. The complete data have been published by Ballard and Nelson (13).

Hanging Pellet Method.—Pellets of pure methylprednisolone I and methylprednisolone II, 0.50 inch in diameter, were compressed at high pressure. The rates of dissolution in deionized water at 37° were determined by the method of Nelson (7), with the exception that instead of determining weight loss, the fluid surrounding the pellet was assayed for the steroid by ultraviolet spectrophotometry.

Pellet Holder in Wruble Machine.—Pellets of pure methylprednisolone I and methylprednisolone II, 0.375 inch in diameter, were compressed at high pressure. The rates of dissolution in deionized water at 37° were determined as follows. The pellets were held in a polyethylene holder at the center of oval 4-fl. oz. bottles containing 120 ml. of deionized water. The bottles were attached to the wheel of the machine described by Wruble (12). After rotation for different intervals of time, the fluid surrounding the pellet was assayed for steroid by ultraviolet spectrophotometry. The dimensions of the pellet initially and after exposure were determined and the average surface area during exposure to the water calculated. The weight of methylprednisolone in solution, which had been released by the pellet, was divided by the average surface area during exposure. The latter values were plotted against time of exposure to the water. The points were fitted by the method of "least squares." The intercepts and slopes (rates) of the lines are shown in Table I. Experiments were made with the wheel rotating at 6 r.p.m. and at 12 r.p.m.

Pellet Holder Method in the Machine of Souder and Ellenbogen (11).—The experiments were performed similarly as described above for the Wruble machine, except the machine described by Souder and Ellenbogen (11) was used in place of the Wruble machine. The bottles were rotated at 40 r.p.m. during exposure in a constant temperature bath held at 37°.

Estimation of Film Thickness.—Under the conditions of our *in vitro* experiments we can substitute D/h for k in Eq. 3 shown in the Appendix, where D is the diffusion coefficient of methylprednisolone in water and h is the diffusion layer or film thickness. Higuchi, Rowe, and Hiestand (14) have estimated D for methylprednisolone in water at 25° to be 4.7×10^{-6} cm.^{2} sec.$^{-1}$ which is equivalent to 1.8×10^{-2} cm.^{2} hr.$^{-1}$ at 37°. Estimations of D/h and h in our experiments, using the latter value, are shown in Table II.

DISCUSSION OF RESULTS

Effect of Intensity of Agitation.—The data in Table I and Fig. 2 indicate that when the intensity of agitation, or velocity of dissolution medium across the dissolving solid, was of a low order of magnitude, then a significant difference in dissolution rates was observed for methylprednisolone forms I and II. When the speed of rotation of the wheel in the Wruble machine was increased from 6 to 12 r.p.m., the rates of dissolution of the two polymorphs became equal within the errors of measurement. Rotation at 40 r.p.m. in the machine described by Souder and Ellenbogen (11) also led to no observable significant difference in rates of dissolution. When implanted in rats the pellets of the two polymorphs lost weight at significantly different rates. These data then indicate that *in vitro* tests in which there is a relatively low intensity of agitation, or only natural convection, correlate best with the *in vivo* implantation data.

TABLE I.—RESULTS OF DISSOLUTION RATE EXPERIMENTS PERFORMED WITH CONSTANT SURFACE PELLETS OF METHYLPREDNISOLONE POLYMORPHS I AND II BY AN *In Vivo* AND SEVERAL *In Vitro* METHODS

Test[a]	Methyl-prednisolone Polymorph	95% Confidence Interval of the Intercept, mg./cm.²	95% Confidence Interval of the Rate, mg./cm.²/hr.	Percentage Difference in Rates[b]	Significance of Difference Between Rates by *t*-test
Pellet implants in rats (13)	I	... ...	0.0179 ± 0.0022	51.1	Sig. 1% level
	II	... ...	0.0302 ± 0.0034		
Hanging pellet method (7)	I	0.077 ± 0.081	0.091 ± 0.021	41.7	Sig. 1% level
	II	0.066 ± 0.119	0.139 ± 0.030		
Pellet holder method in Wruble machine (12), 6 r.p.m.	I	0.029 ± 0.083	0.203 ± 0.027	26.5	Sig. 2% level
	II	0.026 ± 0.123	0.265 ± 0.047		
Pellet holder method in Wruble machine (12), 12 r.p.m.	I	0.048 ± 0.028	0.276 ± 0.011	−0.4	No sig. diff.
	II	0.203 ± 0.115	0.275 ± 0.045		
Pellet holder method in machine of Souder and Ellenbogen (11), 40 r.p.m.	I	0.100 ± 0.039	0.630 ± 0.034	4.0	No sig. diff.
	II	0.118 ± 0.180	0.656 ± 0.156		

[a] Intensity of agitation increasing in descending order. [b] Percentage difference in rates = $\frac{\text{rate for methylprednisolone II} - \text{rate for methylprednisolone I}}{\text{average of rates of methylprednisolone I and II}} \times 100$.

TABLE II.—ESTIMATIONS OF FILM THICKNESSES (h)

In Vitro Method	Methylprednisolone I		Methylprednisolone II	
	D/h,[a] cm. hr.$^{-1}$	h,[b] cm.	D/h,[a] cm. hr.$^{-1}$	h,[b] cm.
Hanging pellet method (7)	0.165	0.11	0.208	0.087
Pellet holder method, Wruble machine (12), 6 r.p.m.	1.73	0.010	1.88	0.0096
Pellet holder method, Wruble machine (12), 12 r.p.m.	2.35	0.0077	1.95	0.0092
Pellet holder method, machine of Souder and Ellenbogen (11), 40 r.p.m.	5.37	0.0034	4.65	0.0039

[a] Obtained by dividing the rate of dissolution by the solubility of methylprednisolone I or II. The latter values are 0.1174 and 0.141 mg. ml.$^{-1}$ at 37°, respectively. [b] Obtained by dividing D, namely 1.8×10^{-2} cm.² hr.$^{-1}$, by the suitable value of D/h.

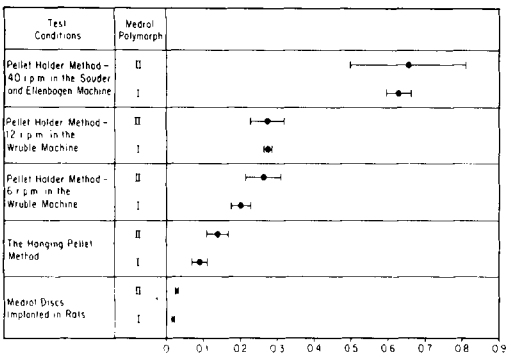


Fig. 2.—Dissolution rates and their 95% confidence intervals for methylprednisolone polymorphs I and II determined under various conditions.

The implications of these results with respect to *in vitro* tests for timed release or prolonged action oral products are important. Before an *in vitro* test is accepted as a routine procedure to be applied to a wide variety of products, one should be sure that the test can differentiate differences between lots of a given product and between different products which do show a difference *in vivo* by their intended route of administration. Otherwise the control test is just a routine operation without any meaning.

Film Thicknesses.—Parrott, Wurster, and Higuchi (2) calculated a diffusion layer or film thickness of 3.28×10^{-3} cm. for benzoic acid dissolving in pure water under their experimental conditions. This is very comparable to the values of 3.4×10^{-3} and 3.9×10^{-3} cm. which we estimated for methylprednisolone I and II, respectively, when the $^3/_8$ inch pellets in our tests were held in the center of a 4-fl. oz. bottle containing 120 ml. of water rotated in the machine described by Souder and Ellenbogen (11). Under the usual conditions in which this machine is used to test timed release or prolonged action products, the effective film thickness would be much less and the intensity of agitation much greater since the tablets or granules are allowed to fall freely through the fluid during the test and are not held rigidly in the center of the bottle as in our tests. Hence, one might expect much greater difficulty in distinguishing between lots of a product by the test used by the Pharmaceutical Contact Section (9) than we experienced in our tests.

In the hanging pellet test we estimated a diffusion layer or film thickness of 0.11 and 0.087 cm. for methylprednisolone I and II, respectively. These film thicknesses are 33 and 22 times, respectively, those estimated when the machine of Souder and Ellenbogen was used. When the Wruble machine, rotating at 6 r.p.m., was used in our tests, the film thicknesses estimated were 1.0×10^{-2} and 9.6×10^{-3} cm. for methylprednisolone I and II, respectively. These are about one-tenth the film thicknesses estimated for the hanging pellet tests.

Hence, diminishing the film thickness by increasing intensity of agitation beyond this point led to failure to distinguish between the polymorphs with respect to dissolution rate.

APPENDIX

Some Literature Concerning Kinetics of Dissolution and Effect of Intensity of Agitation.—Hixson and Crowell (8) derived the relationship shown as Eq. 1.

$$dW/dt = kS(C_s - C) \qquad \text{(Eq. 1)}$$

where $-(dW/dt)$ is the rate of change of weight of the dissolving substance or velocity of solution of a dissolving substance and dW/dt is the rate of appearance of the dissolving substance in the dissolution medium, k is a constant with dimensions distance/time, S is the surface area of the dissolving substance, C_s is the concentration of a saturated solution of the dissolving substance in the dissolution medium, and C is the concentration of the dissolving substance in the dissolution medium at time t. The value of the constant k has been shown (8) to be dependent upon the temperature, the structure of the surface of the dissolving substance, the arrangement of the apparatus, and the rate of stirring or agitation (or the velocity of the dissolution medium across the surface of the dissolving substance).

When C is always negligible in comparison to C_s, Nelson (15) showed that Eq. 1 reduces to

$$dW/dt = kSC_s \qquad \text{(Eq. 2)}$$

Integrating Eq. 2, with S considered a constant, yields

$$W = kSC_st \qquad \text{(Eq. 3)}$$

Polli (4) reviewed the literature concerning the effect of agitation speed on the velocity of heterogeneous reactions. Investigators have usually reported the following relationship

$$K = aV^b \qquad \text{(Eq. 4)}$$

where K is a constant similar to k in Eq. 1, V is the velocity of agitation, and a and b are constants. In accordance with the Nernst-Brunner theory, the value of the exponent b has been found to be 1, or approximately 1, when the reaction taking place was diffusion controlled. On the other hand, for reactions controlled by the rate of the interfacial reaction, values for b were found to approach zero. After studying the dissolution of inorganic salts, Davion (5) reported he obtained a value of 0.5 for the constant b. Hence, in terms of the symbols used above Davion found

$$k = a\sqrt{V} \qquad \text{(Eq. 5)}$$

Van Krevelen and Krekels (16) found a similar relationship for the rate of dissolution of solid substances when they are contacted with a liquid current in the form of a granular bed. Hence a plot of k *vs.* V would pass through the origin and be parabolic with the slope of the line approaching zero, but always positive as V increases.

Linton and Sherwood (6) and Davion (5) applied the methods of investigation applicable to heat transfer to the kinetics of dissolution. The former authors measured the rate of solution of slightly soluble fused salts from the inner walls of cast tubes through which water was forced both in streamline and turbulent flow at velocities from 0.5 to 500 cm./sec. Data were also obtained on the rate of solution of cast plates, cylinders, and spheres of the same materials with water velocities of 5 to 90 cm./sec. For streamline flow in tubes their data checked reasonably well with the Leveque equation, which is

$$\frac{C - C_0}{C_s - C_0} = 5.5\left(\frac{R}{D_L\rho L}\right)^{-2/3} \qquad \text{(Eq. 6)}$$

where C is concentration of exit stream in Gm./ml., C_0 is the concentration of inlet stream, C_s is concentration at saturation, R is the flow rate of the fluid in Gm./sec., D_L is molecular diffusivity in cm.^{2}/sec., ρ is the density in Gm./ml., and L is the length of the tube in cm. Their results supported the assumption of parabolic flow, molecular diffusion, and saturation at the solid-liquid interface.

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Prediction of Stability in Pharmaceutical Preparations IX

Solution Degradation and Chemical Assay of the Antibiotic Actinospectacin

By EDWARD R. GARRETT† and GERALD R. UMBREIT

The kinetics of degradation of the antibiotic actinospectacin[1] in solution have demonstrated that the material is unstable in the alkaline region and that its degradation is catalyzed by buffer anions such as borate, phosphate, and carbonate. Equations have been obtained to characterize degradation as a function of hydroxyl ion concentration and temperature. A chemical assay has been devised based on the formation of the copper complex of a derived dithiocarbamic acid which is extractable and spectrophotometrically measurable in organic solvent. Optimum conditions are described to measure the unique secondary amine functions of the active antibiotic and the change in solubility characteristics of the copper complex of the derived dithiocarbamic acids. Bioassay has been correlated with this chemical assay and optimum conditions defined for its use.

ACTINOSPECTACIN sulfate (1) is a new, therapeutically effective antibiotic, active *in vitro* and *in vivo* against a variety of Gram-positive and Gram-negative organisms (2). It is a material of apparent molecular formula $C_{14}H_{26}N_2O_7$ which is isolated as the hydrated sulfate, and in that form has an apparent molecular weight of 506 (3). It has two secondary amine groups and the two pKa values assigned to these groups are 6.95 and 8.70 (3). Its high water solubility and low organic solubility indicate a hydroxylated compound.

This paper reports on studies designed to establish a chemical assay based on the presence of secondary amine groups, to determine the stability of actinospectacin as a function of pH and temperature, to compare chemical and biological assay procedures, and to predict the possible nature of the solution degradation and the conditions for maximum stability.

EXPERIMENTAL

General Procedure.—The isolation and characterization of the actinospectacin sulfate used has been reported (1–3). Kinetic studies were conducted principally at 30 and 60° in 0.1 *M* hydrochloric acid, acetate buffers (pH 4.65); phosphate, bicarbonate, and borate buffers (pH 7–11); and in varying concentrations of sodium hydroxide. No significant degradation of actinospectacin was observed by chemical or biological assay in 0.1 *M* hydrochloric acid and at pH 4.65 for over 43 days at 30°. Alkaline degradation, however, was significant and studied in considerable detail.

Aliquots were taken at recorded intervals from the actinospectacin solutions maintained in constant temperature baths at ±0.05°.

The aliquots were immediately neutralized and then diluted 1:4 with 0.1 *M* pH 7.85 phosphate buffer for microbiological assay, or diluted 1:4 with 0.2 *M* sodium hydroxide – 0.4 *M* acetic acid buffer for chemical assay, except for the phosphate and acetate buffered solutions which were assayed without dilution. The resultant solution submitted for microbiological assay was 0.08 *M* in pH 7.85 phosphate buffer and contained 450 mcg./ml. as based on undegraded actinospectacin. The resultant solution assayed chemically contained 400 mcg./ml. as based on undegraded actinospectacin.

Estimates of heats of activation of alkaline degradation were obtained by kinetic studies in 0.1 *M* sodium hydroxide at several temperatures.

The complete conditions are given in Table I for the kinetic runs and the estimated apparent first-order rate constants.

Chemical Assay Procedure.—The basis of the chemical assay was the reaction of the secondary amine function of undegraded actinospectacin with carbon disulfide to form dithiocarbamic acid. Addition of a cupric salt gave a complex extractable into organic solvents which could be measured spectrophotometrically (4–6).

The actinospectacin aliquot (1 ml. of an aqueous solution containing 50 to 500 mcg.) was pipetted into a 15-ml. glass-stoppered test tube and 4 ml. of a solution of carbon disulfide, pyridine, and isopropyl alcohol in the ratio 35:25:65 was added. Then, 2 ml. of an 0.0025 *M* cupric chloride solution in water-pyridine, 1:1, was added. The tube was swirled to mix the reactants and allowed to stand at room temperature for 30 ± 5 minutes. Some of the carbon disulfide settled to the bottom.

Subsequently, 3 ml. of water and 3 ml. of benzene were added and the mixture was shaken and inverted to extract the actinospectacin-dithiocarbamate-copper complex. The liquid phases were allowed to separate and 5 ml. of the organic layer was with-

Received June 12, 1961, from the Research Division, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication July 24, 1961.
The authors are greatly indebted to Mrs. Lillian G. Snyder and Mr. Max E. Royer for excellent technical assistance and to Dr. L. J. Hanka and Mr. M. R. Burch and associates for the microbiological assays.
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[1] The trade name of The Upjohn Co. for the antibiotic actinospectacin is Trobicin.

TABLE I.—CONDITIONS AND RATE CONSTANTS (k in sec.$^{-1}$) FOR THE APPARENT FIRST-ORDER DEGRADATION OF ACTINOSPECTACIN SULFATE AS DETERMINED BY CHEMICAL ASSAY

Kinetic Run No.	°C.	Buffer Composition after Correction for Neutralization of Actinospectacin Salt		Molarity of Actinospectacin in Degrading Solution[a]	Average Observed pH for Run[b]	10^4k sec.$^{-1}$
		(NaOH)				
1	30.2	0.002 *M*	...	0.0040	10.14	1.34
2	30.2	0.012	...	0.0040	11.68	7.90
3	30.2	0.017	...	0.0040	...	10.1
4	30.2	0.042	...	0.0042	...	13.5
5	30.2	0.092	...	0.0040	...	16.2
6	30.2	0.142	...	0.0041	...	19.7
7	30.2	0.192	...	0.0040	...	23.2
8	30.2	0.242	...	0.0040	...	24.6
9	30.2	0.292	...	0.0042	...	26.7
10	40.2	0.092	...	0.0040	...	47.6
11	49.9	0.092	...	0.0040	...	116.0
		(K_2CO_3)	($KHCO_3$)			
12	60.0	0.092 *M*	0.008 *M*	0.0040	10.61	120.0
13	60.0	0.060	0.040	0.0040	9.84	39.5
14	60.0	0.026	0.074	0.0040	9.23	13.6
		(NaH_2BO_3)	(H_3BO_3)			
15	60.0	0.0372	0.0128	0.0040	9.20	1.13
16	60.0	0.0263	0.0237	0.0040	8.82	0.596
17	60.0	0.0120	0.0380	0.0039	8.16	0.285
18	60.0	0.0042	0.0458	0.0040	6.88[c]	0.113
		($NaKHPO_4$)	(KH_2PO_4)			
19	60.0	0.0427	0.0073	0.0040	7.22	0.447
20	60.0	0.0284	0.0216	0.0040	6.71	0.129
21	30.2	0.0973	0.0062	0.0020	8.13	0.007[d]

[a] Calculated from assumed molecular weight, 505.87. [b] Maximum range of 0.1 pH unit for duration of study. pH was read at the temperature of the run. [c] Was prepared to be 7.7 pH. [d] Because of the few points available for this run, this represents only the magnitude of the rate.

drawn, dried over 0.3 Gm. of anhydrous sodium sulfate, and transferred to a cell. Spectrophotometric measurement at 470 mμ with a Beckman model B spectrophotometer was made against a reagent blank prepared in the same manner but without antibiotic. A typical absorption band for the copper-dithiocarbamate complex of actinospectacin is given in Fig. 1. A standard curve of absorbance of organic phase extracted antibiotic *vs.* actinospectacin sulfate concentration was prepared in the same manner and followed Beer's law (see Fig. 2).

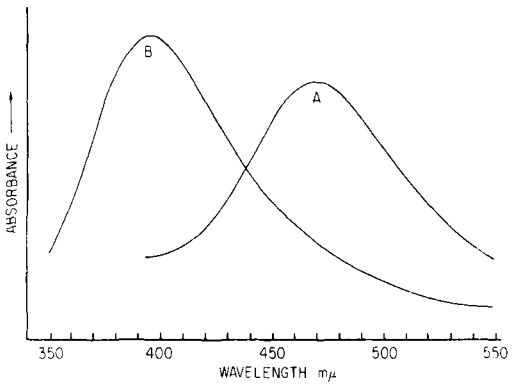


Fig. 1.—Typical visible absorption bands for the copper-dithiocarbamate complex of actinospectacin extracted in the upper organic phase (A) and for the copper dithiocarbamate complex of degraded actinospectacin unextracted from the lower aqueous phase (B).

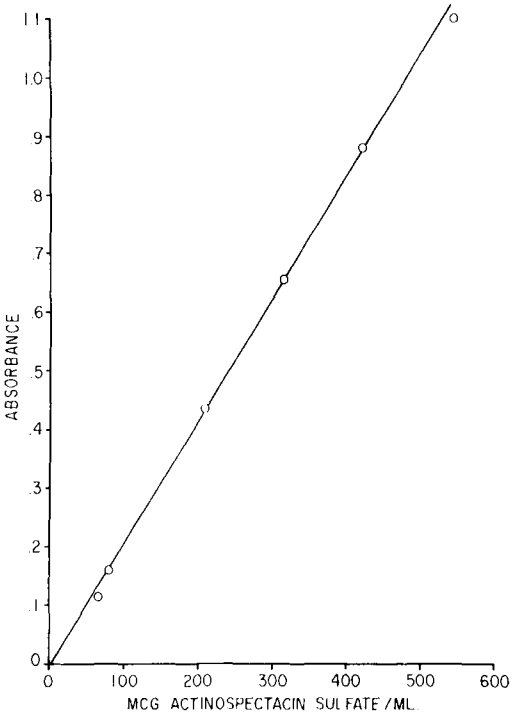


Fig. 2.—Standard curve for the 470 mμ absorbance of the complex, actinospectacin-dithiocarbamate-Cu^{++}, extracted into the upper organic phase against the initial concentration of actinospectacin-sulfate in aqueous solution.

As the alkaline degradation proceeded, color attributable to complex increased in the aqueous phase. At the beginning of a reaction, colored complex was totally soluble in the organic layer; at the end of a reaction, colored complex was totally soluble in the aqueous layer. The reaction could also be followed by the increasing appearance by spectrophotometry of colored reaction complex remaining in the aqueous phase after extraction (see Fig. 1).

Effect of Conditions on Absorbance of Colored Complexes.—A phenomenon occurred with several of the kinetic runs that warranted further investigation. The absorbance of the actinospectacin-dithiocarbamate-copper complex extracted in the organic phase at zero time for several of the kinetic runs did not agree with the values expected from the standard curve (see Fig. 2). In several instances the apparent absorbances were significantly less.

Of course, this did not affect the estimation of the apparent first-order rate constants for the decomposition of actinospectacin but it did raise the question as to the validity of the assay. The assay's reliability was checked by its excellent replication under the same conditions.

To investigate this phenomenon, aliquots of aqueous actinospectacin solutions of the same antibiotic concentration with various amounts of other materials were chemically assayed. The resultant apparent absorbances of the complex extracted into the organic phases were plotted as a function of the concentrations of these other materials present. These addends were similar to the components of the buffers used in the kinetic runs, i. e., acetic acid, sodium acetate, hydrochloric acid, and potassium chloride (see Fig. 3).

Chloride and acetate ions had no effect when the source of these ions was a neutral salt, but the corresponding acids gave a definite decrease in the color intensity of the actinospectacin-dithiocarbamate-copper complex extracted in the organic phase. In contrast, no decrease in the intensity of color from the complex with di-*n*-butylamine was noted when excess acetic or hydrochloric acid was added.

Characterization of Actinospectacin-Dithiocarbamate-Copper Complex.—An 0.7701-Gm. sample of actinospectacin sulfate was dissolved in 50 ml. pyridine, 25 ml. water, and 50 ml. isopropyl alcohol. This was titrated by the method of Critchfield and Johnson (4) with 31.15 ml. 0.100 *M* sodium hydroxide to phenolphthalein end point. Then 5 ml. of carbon disulfide was added and 30.40 ml. 0.100 *M* sodium hydroxide was necessary to reach the phenolphthalein end point again. A simultaneously prepared blank took 0.2 ml. titer to neutralize and another 0.2 ml. subsequent to the addition of carbon disulfide. Thus, 3.09 meq. of sulfuric acid and 3.02 meq. of dithiocarbamic acid (R_1R_2N—CS—SH) were found which calculates to 1.023 sulfuric acid per molecule and is consistent with the characterization of the original actinospectacin sulfate used, $C_{14}H_{26}N_2O_7 \cdot 1.12H_2SO_4 \cdot 3.43H_2O$. mol. wt. 505 (3). The pKa's were 7.0 and 8.8. It also demonstrates the formation of two dithiocarbamic acid groups per molecule.

The actinospectacin-dithiocarbamate-copper complex in the organic phase was isolated from the separated organic layer by evaporation of the solvent at room temperature. The infrared spectrum of this material was nearly identical with that of the original actinospectacin sulfate and thus indicated that no apparent degradation had occurred in this isolation procedure.

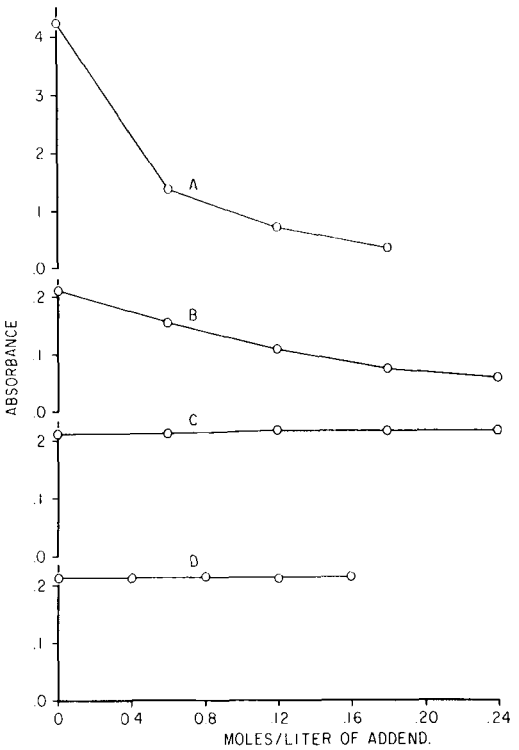


Fig. 3.—Effect of addends in original aqueous solution on 470 mμ absorbance of actinospectacin-dithiocarbamate-Cu^{++} complex extracted into upper organic phase

Curve	Initial Amount of Actinospectacin (mcg.)	Addend
A	208	HCl
B	104	$HC_2H_3O_2$
C	104	$NaC_2H_3O_2$
D	104	KCl

This material had 11.68% ash. If it is assumed that the nonhydrated form of the actinospectacin-dithiocarbamate-copper complex, $C_{14}H_{24}N_2O_7(CS_2)_2$-Cu, mol. wt. 547, is extracted in the organic phase, then the theoretical per cent copper for a copper to complex ratio to be 1:1 would be 11.5% as copper. If the ash were copper oxide, the theoretical for a 1:1 ratio would be 14.4% on the assumption of a nonsolvated sample. The experimentally obtained value is inconsistent with a 2:1 ratio where a 25.9% ash would be expected and thus confirms a 1:1 ratio of copper to partially solvated complex.

Samples of 0.304 mg. actinospectacin sulfate were treated in the manner described by the chemical assay procedure with slight modifications. The concentration of cupric chloride was varied. The absorbances of the colored actinospectacin-dithiocarbamate-copper complex in the organic layer attained the following values for a stated copper: actinospectacin sulfate molar ratio: 0.214, 1:1;

0.409, 2:1; 0.427, 3:1; 0.427, 5:1; 0.410, 7:1; 0.385, 10:1; 0.061, 20:1; and 0.055, 50:1.

The colored complex extracted into the organic phase achieved a maximum at *ca.* 4:1 ratio of reactants, copper to actinospectacin. With additional copper concentrations, the amount of 1:1 colored complex drastically decreased, proving the formation of a noncolored actinospectacin-dithiocarbamate-copper reaction product of a copper:actinospectacin ratio of 2:1 or more.

Characterizations of Degraded Actinospectacin and Its Dithiocarbamate Complex.—A 25-mg. sample of actinospectacin sulfate was degraded in 25 ml. 0.1 *M* sodium hydroxide at 25° for 24 hours, which is greatly in excess of the time necessary for complete loss of biological activity and identification as actinospectacin by the chemical assay. The resultant solution was neutralized so that the original material was at a 1:1 dilution with water. A series of aliquots of x ml., varying from 0.000 to 1.000 ml. by 0.050-ml. increments, were transferred to test tubes. A solution of cupric chloride of a molarity equivalent to the neutralized, diluted reaction solution was prepared. Then, $(1 - x)$ ml. of this cupric chloride solution was added to each test tube containing x ml. of the degraded actinospectacin solution according to the method of continuous variations (7). The chemical assay reagents and solvents were added as in the manner previously described. The absorbances were read in the Cary recording spectrophotometer, model 11, from 350–500 mμ using the tungsten lamp. The observed maximum was 462.5 mμ in the aqueous phase. The organic layer was colorless. A plot of the absorbance at this wavelength maximum against the mole fraction of degraded actinospectacin in degraded actinospectacin + copper gave a clear maximum at 0.5 mole fraction. This demonstrates that the 462.5 mμ maximum is defined by a 1:1 ratio of copper to the dithiocarbamate of degraded actinospectacin (7). When the ratio of copper to degraded actinospectacin was 4:1 or greater, a 390 mμ maximum started to appear. When additional copper was added so that the ratio was 10:1 in favor of the copper, the wavelength of the maximum absorbance in the aqueous layer shifted to 391 mμ (Fig. 1) and the 463 mμ maximum disappeared. This clearly indicates that a 2:1 complex of copper to the dithiocarbamate of degraded actinospectacin is formed at higher copper concentrations.

Infrared spectroscopy could not differentiate between actinospectacin and its degradation product. Nuclear magnetic resonance indicated that a doublet assigned to $-\underset{|}{CH}-CH_3$ in actinospectacin has been transformed to two sets of doublets in degraded actinospectacin; a new $-\underset{|}{CH}-CH_3$ has appeared. The area of the resultant two $-\underset{|}{CH}-CH_3$ groups is equivalent to the area attributable to one in the original actinospectacin. The same functional groups exist in actinospectacin and degraded actinospectacin except that all peak values are shifted about 10 cycles higher in the latter case. However, the peaks are superimposable when shifted, thus indicating slight changes in geometry.

The sulfate salt of the isolated degraded actinospectacin, dried in the same manner as actinospectacin sulfate at high vacuum and room temperature, had an equivalent weight of 252, so that its molecular weight of 504 is the same as actinospectacin sulfate. Its pKa's were 7.1 and 8.9.

Optical Activity and Degradation.—A solution of actinospectacin was prepared so that it was 0.05 *M* in actinospectacin free base and 0.10 *M* in sodium hydroxide. The optical rotation was read in a 1-dcm. tube at intervals of time at 400 mμ. The initial reading of +0.020 changed with time and asymptotically approached −1.700. The logarithm of the difference of the optical rotation at any time, t, and the asymptotic value was plotted against the time, t, and was a straight line. The resultant rate constant for the degradation as followed by this stereochemical transformation at 25° in 0.10 *M* sodium hydroxide was 9.73×10^{-4} sec.$^{-1}$, a half life of 11.8 minutes. The extrapolated initial rotation was +0.0188.

It will later be shown (from Eq. 13) that the degradation at 25° in 0.092 *M* sodium hydroxide by chemical assay is 9.55×10^{-4} sec.$^{-1}$.

Biological Assay Procedure.—The biological assay was a plate-disk assay against *Klebsiella pneumoniae* (8) and was run simultaneously with the chemical assay on aliquots of degrading actinospectacin. When anomalies resulted in bioassay dependent on whether or not the aliquot was diluted with phosphate buffer, a special study on the effect of pH 7.85 phosphate buffer concentration on apparent antibiotic potency was undertaken.

Actinospectacin (125.4 mg.) was weighed into a 50-ml. volumetric flask, diluted to volume with 0.15 *M* sodium hydroxide from the 30° bath, maintained at 30° and assayed at recorded times by the following procedures: *Bioassay Samples.*—Five-milliliter aliquots were pipetted into 10-ml. volumetric flasks containing 0.4 ml. 1.904 *M* hydrochloric acid to "stop" the reaction. One milliliter of neutralized solution was then pipetted into each of five 5-ml. volumetric flasks and diluted to volume with the appropriate concentration of pH 7.85 phosphate buffer to make the resultant solutions 0.16, 0.10, 0.05, 0.025, and 0.01 *M* in phosphate buffer. One milliliter of each of the five resultant solutions was transferred to an assay bottle and submitted for microbiological assay without further dilution. The concentration of the final solution prior to alkali degradation was 465 mcg./ml.

Chemical Assay Samples.—Aliquots (0.8 ml.) were pipetted into a 5-ml. volumetric flask containing *ca.* 4 ml. of an equal mixture of 0.2 *M* sodium hydroxide and 0.4 *M* acetic acid, and diluted up to 5 ml. with the same buffer mixture. One milliliter of the resultant solution was assayed by the chemical assay procedure. The concentration of the final solution prior to alkali degradation was 402 mcg./ml.

The results showed that the molarity of 7.85 pH phosphate buffer significantly affected the estimate of the apparent antibiotic concentration from microbiological assay as compared with the concentration from chemical assay (see Fig. 4). It was clearly shown that if the 7.85 pH buffer concentration was maintained above 0.05 *M* in the solution to be microbiologically assayed, a 1:1 correspondence with the chemical assay resulted for material degraded in 0.15 *M* sodium hydroxide at

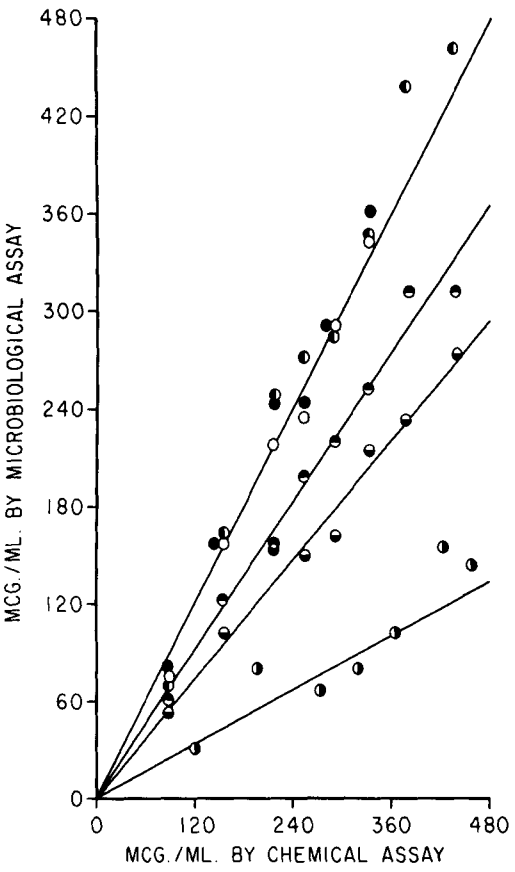


Fig. 4.—Effect of pH 7.85 phosphate buffer concentration on the microbiological assay of actinospectacin. The aliquots assayed were sampled at various times from an actinospectacin solution degrading in 0.15 *M* sodium hydroxide at 30°. ○, 0.160; ●, 0.100; ◐, 0.050; ◓, 0.025; ◒, 0.010; ◑, 0.000 *M* of phosphate buffer.

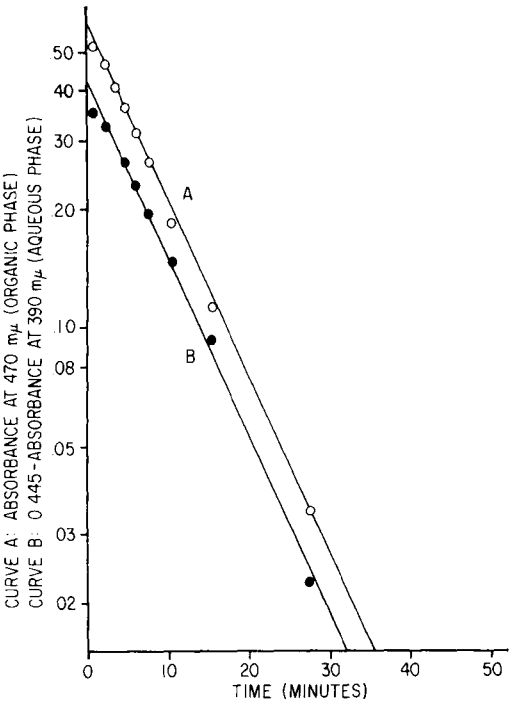


Fig. 5.—Apparent first-order rate plots for the degradation of actinospectacin in 0.15 *M* sodium hydroxide aqueous solution at 30°. Curve A is derived from the absorbance at 470 mμ of the actinospectacin-dithiocarbamate-Cu^{++} complex extracted in the upper organic phase and is a measure of the disappearance of the antibiotic. Curve B is derived from the absorbance at 390 mμ of the dithiocarbamate-Cu^{++} complex of degraded actinospectacin and is a measure of the appearance of the product of degradation.

30°. Thus, in all attempted correlations of bioassay with color assay, this minimum phosphate buffer concentration was maintained. The attempted correlations were conducted on the series of alkali-degrading actinospectacin solutions where the statistics of regression are given in Table II.

RESULTS AND DISCUSSION

Apparent First-Order Rates of Alkaline Degradation.—The alkaline degradation of actinospectacin in solution is the major route of antibiotic decomposition. At a given pH or hydroxyl ion concentration, it is apparently first order

$$\log A = \frac{-kt}{2.303} + \log A_0 \qquad \text{(Eq. 1)}$$

where A is the absorbance at 470 mμ of the organic phase complex (with Cu^{++} and CS_2) at any time, t, and A_0 is the absorbance at initial time, t_0. Figure 5 demonstrates the linearity of such semilogarithmic plots. The extraordinary phenomenon of the Cu^{++} CS_2 complex of the degraded actinospectacin being completely water soluble is demonstrated by curve B in Fig. 5, a line parallel to curve A for the decrease of actinospectacin complex absorbance in the organic phase. The rate of degraded actinospectacin complex appearance in the aqueous phase completely parallels the rate of actinospectacin complex disappearance from the organic phase. This former plot is based on the expression

$$\log (A'_\infty - A') = \frac{-kt}{2.303} + \log A'_\infty \qquad \text{(Eq. 2)}$$

where A' is the aqueous phase absorbance at 390 mμ (the $\lambda_{max.}$ of the degraded complex absorbance in the water phase) at any time, t, and A'_∞ is the final or asymptotic value of this absorbance, 0.445.

Formation of Dithiocarbamates and the Possible Nature of the Complexes.—The presumed reactions (4, 5) in the formation of dithiocarbamates and their copper complexes are

$$R_1R_2NH + CS_2 \rightleftharpoons R_1R_2N{-}CS{-}SH \qquad \text{(Eq. 3)}$$

$$R_1R_2N{-}CS{-}SH + Cu^{++} \rightleftharpoons R_1R_2N{-}CS{-}SCu^{+} + H^{+} \qquad \text{(Eq. 4)}$$

$$R_1R_2N{-}CS{-}SCu^{+} + R_1R_2N{-}CS{-}SH \rightleftharpoons (R_1R_2N{-}CS{-}S)_2Cu + H^{+} \qquad \text{(Eq. 5)}$$

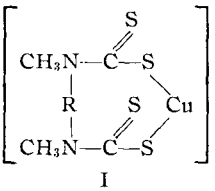
TABLE II.—STATISTICS OF REGRESSIONS OF BIOASSAYS (y) ON CHEMICAL ASSAYS (x) FOR ACTINOSPECTACIN DEGRADING IN ALKALINE SOLUTION AT 30.2°

Molarity of NaOH	No. of Points Used	Line Forced through Origin; $y=mx$				Line of Best Fit; $y=mx+b$						
		m	σm	σy	$t\sigma m$	m	σm	b	σb	σy	$t\sigma m$	$t\sigma b$
0.02	7	1.05	0.039	27.7	0.095	1.22	0.059	−48.7	15.8	17.8	0.151	40.6
0.044	6	0.898	0.038	25.0	0.099	0.846	0.099	14.9	26.2	26.8	0.274	72.6
0.047	7	0.816	0.046	36.0	0.112	0.776	0.116	13.1	34.4	38.8	0.298	88.5
0.082	7	0.854	0.031	26.6	0.075	0.934	0.091	28.1	29.9	26.9	0.235	76.9
0.115	6	1.05	0.038	22.0	0.098	1.08	0.119	− 9.50	28.2	24.3	0.331	78.4
0.190	8	0.891	0.048	35.8	0.113	0.821	0.120	20.4	31.7	37.4	0.293	77.5
0.200	8	0.852	0.021	15.9	0.049	0.959	0.031	−31.4	8.46	9.45	0.077	20.7

$$(R_1R_2N{-}CS{-}S)_2Cu + Cu^{++} \rightleftharpoons 2R_1R_2N{-}CS{-}SCu^{+} \quad \text{(Eq. 6)}$$

The introduction of two CS_2 per molecule was demonstrated experimentally by the titration method of Critchfield and Johnson (4).

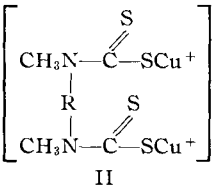
When these series of reactions and the previously described procedures were used to follow the rates of degradation of actinospectacin there was, initially, no colored complex in the aqueous phase. Finally, after degradation, there was no colored complex in the organic phase. It thus follows that this degradation of actinospectacin modified the amine groups in a manner that changes an organic phase-soluble complex to a water-soluble one. A possible explanation is an alkaline induced variation of a stereospecific relation of the two nitrogens in actinospectacin. In the case of the organic phase-soluble actinospectacin complex, both dithiocarbamic acid groups in the molecule may be complexed by one cupric ion as per Eqs. 3–5, giving I.



I

Actinospectacin-2CS_2-Cu Complex
Organic Phase Soluble; $\lambda_{max.}$ = 470 mμ

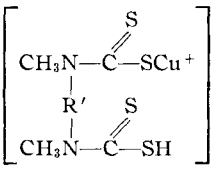
The 1:1 = Cu:actinospectacin nature of this complex, I, was shown experimentally by the agreement of the per cent ash as CuO with the theoretical for 1:1. Addition of excess cupric ion, 20:1 = Cu:actinospectacin, dramatically decreased the absorbance extracted into the organic phase, proving the formation of a new complex consistent with Eq. 6 so that we have II



II

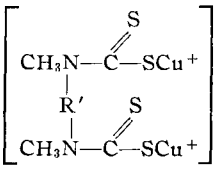
Actinospectacin-$(CS_2Cu^{+})_2$ Complex

For degraded actinospectacin a chelated copper ion may not be sterically possible as in I so that a charged water-soluble 1:1 complex would be III, a 2:1 complex would be IV.



III

Degraded Actinospectacin-2CS_2-Cu^{+} Complex
Aqueous Phase Soluble; $\lambda_{max.}$ = 462.5 mμ



IV

Degraded Actinospectacin-$(CS_2Cu^{+})_2$ Complex
Aqueous Phase Soluble; $\lambda_{max.}$ = 390 mμ

By the method of continuous variations (7), III was shown to be a 1:1 = Cu:degraded actinospectacin complex. The new 390 mμ maximum characteristic of IV appeared when excess cupric ion was added in a 10:1 ratio to the degraded actinospectacin.

Infrared and nuclear magnetic resonance spectroscopy could only indicate steric transformation in the alkaline degradation of actinospectacin; they could not identify changes in functional groups.

Comparison of the equivalent weights and pKa's of actinospectacin sulfate and isolated degraded actinospectacin sulfate also could not demonstrate any change in the molecule on degradation.

In addition, the change in optical activity in 0.1 *M* sodium hydroxide confirmed the models proposed. Alkaline degradation of actinospectacin involved a stereochemical transformation and the rates so calculated were identical to the rates by chemical assay.[2]

The variation in the absorbance of the actinospectacin-dithiocarbamate-copper complex extracted into the organic phase when acids were added needs explanation.

The pyridine which is used as part of the solvent mixture for the analytical reaction may be implicated. It partially neutralizes the salt of the secondary amine and helps solubilize the carbon

[2] These experimental facts may not be considered conclusive evidence to negate functional group changes. It is possible that degraded actinospectacin may have enhanced aqueous solubility and that its dithiocarbamate-copper complex may have low organic solvent solubility.

disulfide in the mixed aqueous system. Pyridine has an aqueous pKa = 5.36 (9) so that the resultant apparent pH of the pyridine–isopropyl alcohol–water–carbon disulfide–cupric chloride solution prior to extraction is significantly decreased by the addition of acids (see Fig. 6). The shape of the pH *vs.* molarity of acid curve (Fig. 6) is very similar to the shape of the curve, absorbance *vs.* molarity of acid (Fig. 3).

This implies that the equilibria of Eqs. 4 and 5 are forced to the left by increased hydrogen ion concentration and that the dithiocarbamate-Cu^+ complex is water soluble and does not contribute to the color in the organic layer.

Rate of Degradation as a Function of Alkalinity.—Figure 7 plots the apparent first-order rate constant, k in $sec.^{-1}$, as a function of the [NaOH] solution prepared for degradation. Actually, since the actinospectacin has an apparent molecular weight of 506 and has two amine groups in the salt form, twice the molar concentration of the actinospectacin should be subtracted from the abscissa values to give the true $[OH^-]$ concentration. Generally, actinospectacin = 0.0040 and thus, the given $[NaOH]_{apparent} = [NaOH]_{true} + 0.0080$ in Fig. 7.

The two lines in Fig. 7 represent linear estimates of the variation of apparent first-order rate constants, k in $sec.^{-1}$ at 30.2°, with hydroxyl ion concentration and may be given as

$$k = k_{OH}[OH^-] = 0.0533\ [OH^-] \quad \text{(Eq. 6)}$$

$$[OH^-] < 0.020$$

$$k = k'_{OH}[OH^-] + K = 5.33 \times 10^{-3}\ [OH^-] + 11.6 \times 10^{-4} \quad \text{(Eq. 7)}$$

$$0.020 < [OH^-]$$

The fact that this apparent hydroxyl ion catalysis constant varies as a function of the magnitude of the $[OH^-]$ is a strong indication of a change in the nature of the actinospectacin with hydroxyl ion. The most natural explanation is that the sugar-like antibiotic has one or more ionizable protons of high pKa, *ca.* 12–13, and the anionic form resists hydroxyl ion attack more readily than the neutral molecule.

An alternative method of correlating the apparent first-order rate constant, k in $sec.^{-1}$, for alkaline attack and hydroxyl ion concentration is by a log k *vs.* pOH plot. See Fig. 8 where pOH is calculated from

$$pOH = \log f[NaOH]_{true} \quad \text{(Eq. 8)}$$

where the activity coefficient, f, at 30° is obtained from Harned and Owen (10).

An empirical relation valid over the pOH range 0.6 to 2.2, from Fig. 8, is

$$\log k = -0.376\ pOH - 2.324;\ pOH < 2.2 \quad \text{(Eq. 9)}$$

This is most probably an estimate of

$$k\ [\text{actinospectacin}] = k_{OH}[OH^-][\text{uncharged actinospectacin}] + k'_{OH}[OH^-]\ [\text{anionic actinospectacin}] \quad \text{(Eq. 10)}$$

where from the dashed line of Fig. 8

$$\log k = -pOH + \log k_{OH}^-;\ pOH > 2.2 \quad \text{(Eq. 11)}$$

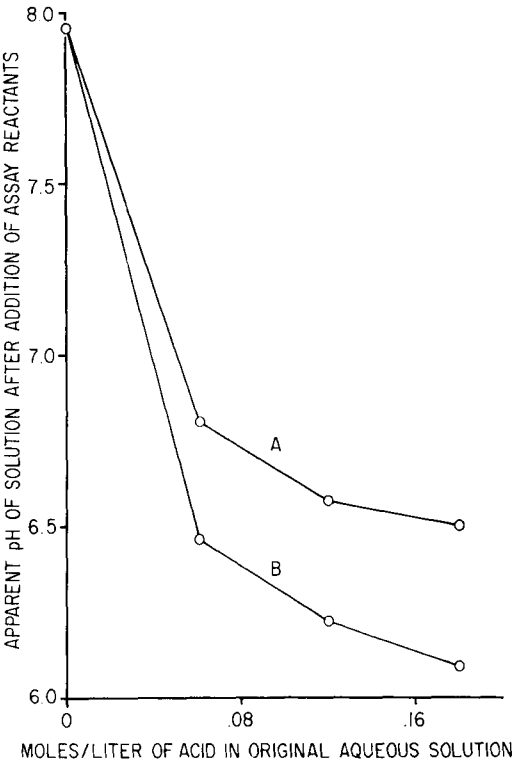


Fig. 6.—Effect of acid added to the original aqueous solution to be assayed for actinospectacin on the apparent pH of the pyridine-isopropyl alcohol-water-carbon disulfide-cupric chloride solution, the composition of the solution just prior to extraction with benzene. Curve A is for acetic acid and Curve B is for hydrochloric acid.

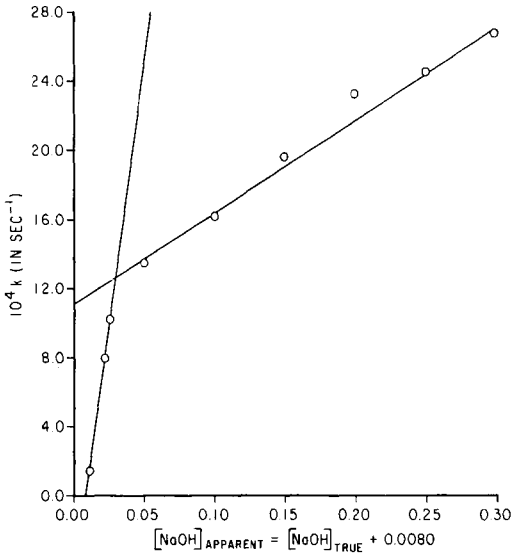


Fig. 7.—Apparent first-order rate constants for the alkaline degradation of actinospectacin as a function of [NaOH] concentration at 30.2°.

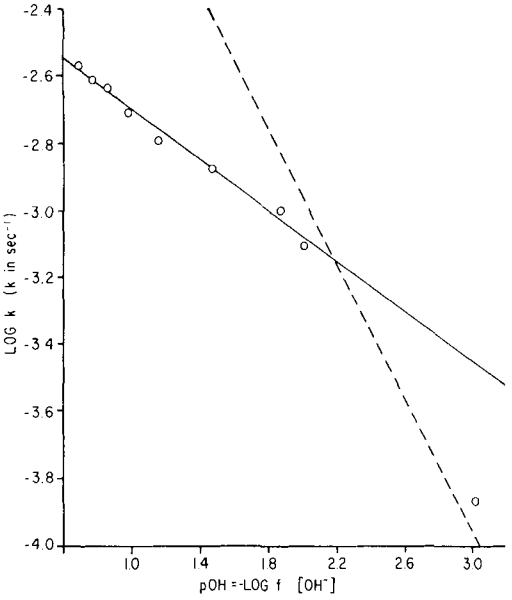


Fig. 8.—Plot of the logarithm of the apparent first-order rate constant for the alkaline degradation of actinospectacin at 30.2° against pOH = log f [OH$^-$] where f is the activity coefficient (9) and [OH$^-$] = [NaOH]$_{True}$. The dashed line has a slope of unity.

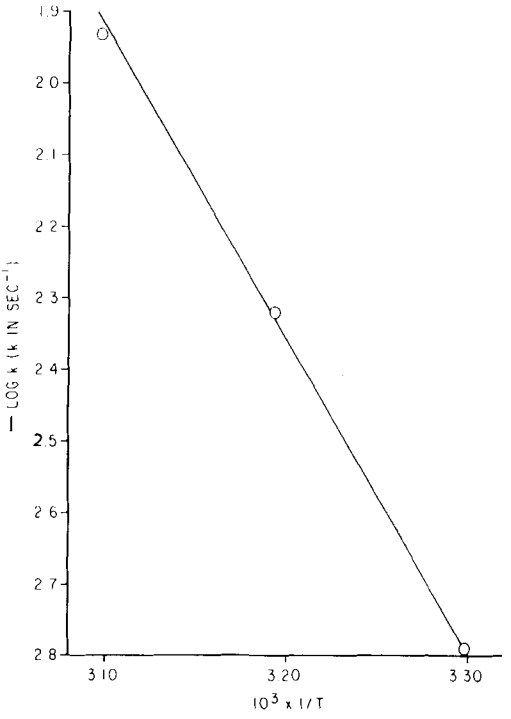


Fig. 9.—Arrhenius plot for the degradation of actinospectacin in 0.092 M sodium hydroxide.

so that log k_{OH^-} = 0.95 or that k_{OH^-} = 0.11 L./M/sec.[3] An estimated pKa of uncharged actinospectacin would be 12.6.[4]

Rate of Degradation as a Function of Buffer Kind.—Since stability in the less alkaline regions had more practical significance and it was indicated that significant degradation did occur at 30° in pH 8 phosphate buffer (half life *ca.* 50 days), an attempt to study degradation in phosphate, borate, and carbonate buffers at 60° was made (see Table I). The rate was not completely dependent on pH but was also a function of the buffer used with carbonate buffers 10 times more catalytic than borate at pH 9 and phosphate *ca.* four times more than borate at pH *ca.* 7.5.

Rate of Alkaline Degradation as a Function of Temperature.—Figure 9 gives the Arrhenius plot for the degradation of actinospectacin at 0.092 M sodium hydroxide. The representative equation is

$$\log k = -4{,}350\,(1/T) + 11.57 \quad \text{(Eq. 12)}$$

where T is absolute temperature and the heat of activation is 20.0 Kcal./mol.

Correlation of Chemical Assay and Microbiological Assay.—During the course of several alkaline degradations, aliquots removed at varying intervals of time were subjected to both microbiological and chemical assays. The statistics of the regression of bioassay *vs.* chemical assay are given in Table II for lines of best fit independent of intercept and for lines of best fit forced through the origin. Of the seven regressions only two, the first and the last listed in Table II, can be stated as having statistically significant nonzero intercepts. Two of the regressions are given in Fig. 10 where the solid lines represent the curve fitted by least squares and the dashed lines are based on adjusted intercepts, $b \pm \sigma_b$, and forced through the mean values, $\bar{y}$ and $\bar{x}$, of the microbiological and chemical assays, respectively. The bracketing of the origin by the intercepts of these dashed curves is apparent. The slope of unit, in general, is demonstrated within the estimated error of the slopes and it can be concluded that a good correspondence exists between the microbiological and the chemical assays.

Prediction of Stability.—Significant solution degradation of actinospectacin occurs in alkaline solution and appears to be a function of hydroxyl ion concentration and the type of buffer anion, *viz.*, carbonate, borate, and phosphate. At 30°, the half life is 50 days in pH 8 phosphate buffer.

On the basis of studies in varying sodium hydroxide concentrations, the half life at 30° in unbuffered aqueous solutions is 1000 days at pH 7.0, 100 days at pH 8.0, and 10 days at pH 9.0.

Experimental studies have shown no significant degradation over two months at pH 4.5 and in 0.1 M hydrochloric acid at 30°.

[3] The reader should not be disturbed at the difference between this value and the one for k_{OH^-} given in Eq. 6. They are defined for different equations. The latter value and [NaOH] are used with Eq. 6 to derive k. The former value and pOH derived from Eq. 8 from consideration of OH$^-$ activity are used with Eq. 11 to derive k. Both methods are valid within experimental error to define k under the conditions specified.

[4] Subsequent titration by D. J. Weber of actinospectacin salt in pyridine with 0.1 N tetra n-butylammonium hydroxide against standard glass-calomel electrodes (methanol saturated with lithium chloride) demonstrated a weakly acidic group that was consistent with this deduced function.

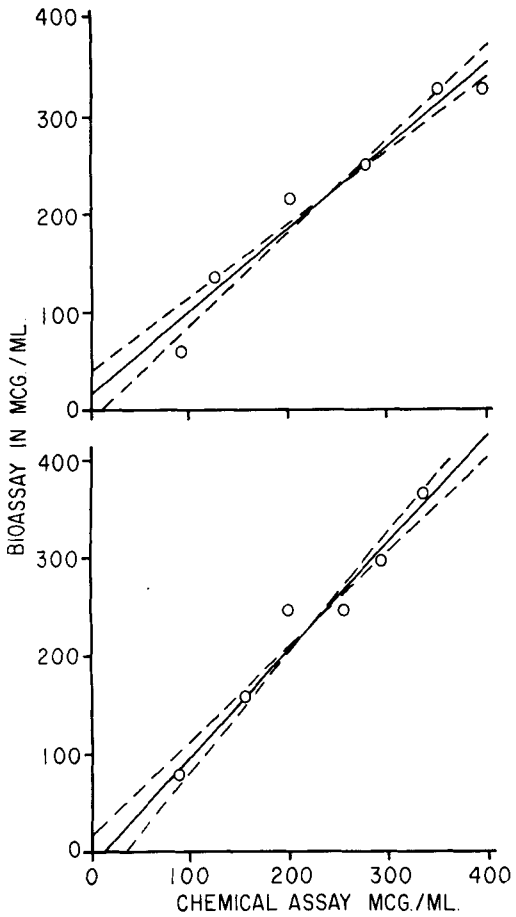


Fig. 10.—Correlation of microbiological plate-disk assay (*Klebsiella pneumoniae*) with chemical assay. The solid line represents the best fit by least squares whereas the dashed lines pass through the mean values and the intercept ± standard deviation of the intercept. The upper curve is based on degradation in 0.044 *M* sodium hydroxide; the lower is based on degradation in 0.115 *M* sodium hydroxide at 30.2°.

SUMMARY

1. Actinospectacin is degraded by specific hydroxyl ion catalysis and the anionic constituents of various buffers: carbonate, phosphate, and borate with decreasing effectiveness in that order. At 30°, the half life is 50 days in pH 8 phosphate buffer, whereas in unbuffered aqueous solution the predicted half life is 1000 days at pH 7.0 and 100 days at pH 8.0 at 30°. Below pH 5, actinospectacin should be stable for all practical purposes.

2. A chemical assay procedure has been devised and applied to the determination of actinospectacin which depends on the unique structure of its secondary amine groups. The formation and extraction of cupric complexed dithiocarbamic acids of actinospectacin permit quantification of undegraded antibiotic by spectrophotometric measurement of visible absorbance in the organic phase. The absorbance of the residual color in the aqueous phase measures degraded antibiotic.

3. The reliability of the bioassay (plate disk *vs. Klebsiella pneumoniae*) was shown to vary with phosphate buffer concentration so that a minimum of 0.05 *M* pH 7.85 phosphate buffer must be present.

4. The bioassay correlates well with the chemical assay.

5. Evidence is presented to demonstrate that the stereospecific relation of the secondary amines in actinospectacin is vital for its biological activity and response to the chemical assay.

6. Complete kinetic expressions have been determined for the alkaline solution degradation of actinospectacin at the various temperatures and a pKa *ca.* 12 for its hydrogen ion dissociation, probably of a sugar-like structure, has been kinetically observed.

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Prediction of Stability in Pharmaceutical Preparations X

Alkaline Hydrolysis of Hydrocortisone Hemisuccinate

By EDWARD R. GARRETT†

The hydrolysis of hydrocortisone 21-hemisuccinate above pH 8 has been studied by constant pH titrations in aqueous solution and appears to involve specific hydroxyl ion catalyzed hydrolysis as the only significant reaction pathway. The apparent heat of activation for specific hydroxyl ion catalyzed hydrolysis when corrected for the heat of ionization of water is unusually low for hemisuccinate esters, 7.0 Kcal./mole. This implicates the configuration and structure of the steroid side chain in the facile hydrolysis of 21-steroid esters. The stability of hydrocortisone 21-hemisuccinate at storage and physiological conditions is predicted.

THE CLINICAL usefulness of hydrocortisone was enhanced by the formation of the water-soluble sodium salt of the 21-hemisuccinate ester (1, 2). The formulation of the water-soluble lyophilized sodium salt of hydrocortisone 21-hemisuccinate[1] permitted facile parenteral administration of hydrocortisone in an homogeneous solution where immediate high levels of circulating hydrocortisone are required.

This paper considers the kinetics of hydrolysis of sodium hydrocortisone hemisuccinate in aqueous solution above pH 7 as studied by constant pH titration. The experimental evidence should permit the prediction of stability of reconstituted solutions and should provide evidence for the understanding of the ester's utilization *in vivo* on parenteral administration.

EXPERIMENTAL

Experimental Procedure for Constant pH Titrations of 0.0165 *M* Hydrocortisone Hemisuccinate—The Cannon di-functional titrator (3) was standardized before each run with Beckman buffer solution which had been equilibrated for a minimum of 5 minutes in the temperature-controlled beaker of the titrator to be at the same temperature as the hydrocortisone hemisuccinate solution to be studied.

Hydrocortisone hemisuccinate which had been dried at 50° in high vacuum was weighed in a small weighing boat on a Gramatic balance. After the Cannon titrator had been standardized as above, the sample was transferred to the temperature-controlled beaker and 15 ml. of 0.0165 *M* sodium hydroxide from the constant temperature bath was added. The sodium hydroxide exactly neutralized the hydrocortisone hemisuccinate. A thick rubber band held a silicone covering over the beaker. Inserted into the silicone gasket were the glass-saturated calomel electrodes and a gas duct to blow nitrogen gently on the surface of the solution in the beaker. No carbon dioxide was absorbed by this system as tested for pH 10 for 1.5 hours.

The starting pH of the solution to be studied was recorded and the titration was then started. In general, the pH of the sodium salt of hydrocortisone hemisuccinate was 6.6. The syringe contained 2.011 *N* sodium hydroxide. The servomotor was used for the first few seconds to raise the pH quickly to the setting of the pH control and then the synchronous motor was used to maintain the solution at this pH. At the end of the run, the temperature and volume of solution in the beaker were recorded. Typical curves of volume of standard alkali consumed *vs.* time are given in Fig. 1. The controlled pH and observed temperature for the various runs are given in Table I. Solvent evaporation occurred, especially at the low rates encountered at low pH values.

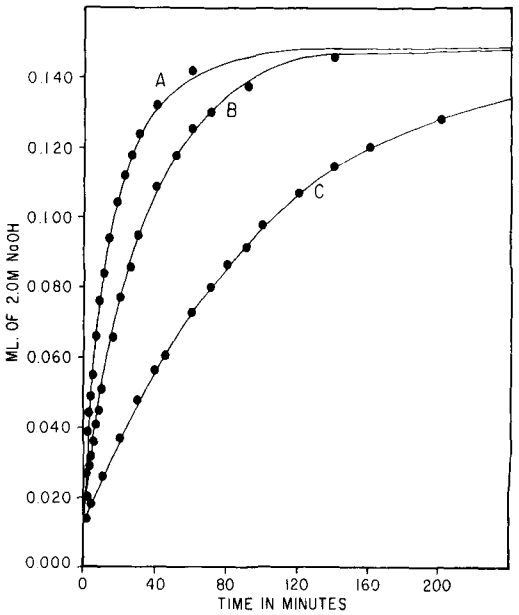


Fig. 1.—Typical curves for the consumption of standard alkali at constant pH by 0.0165 *M* hydrocortisone hemisuccinate at 48°. Curve A is at pH 10.0; curve B is at pH 9.5, and curve C is at pH 9.0.

Received June 19, 1961, from the Research Division, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication July 27, 1961.
The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance.
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[1] The trade name of The Upjohn Co. for hydrocortisone sodium succinate is Solu-Cortef.

TABLE I.—RATE CONSTANTS FOR THE APPARENT FIRST-ORDER HYDROLYSIS OF 0.0165 *M* HYDROCORTISONE HEMISUCCINATE AT VARIOUS TEMPERATURES AND pH VALUES

pH	°C.	10^4k(sec.$^{-1}$) Exptl.		10^4k(sec.$^{-1}$) Calcd.[a]
10	65.2	57[b]	53	50.8
10	64.0	47[b]		49.0
9.5	65.2	15[b]	14	16.0
9.5	63.7	13[b]		15.3
9.0	65.8	7.6[b]		5.16
9.0	65.3	4.1[b]	5.0	5.08
9.0	63.9	3.3[b]		4.88
8.5	65.3	1.3[b]	1.6	1.60
8.5	64.9	2.1[b]		1.60
8.0	65.1	0.88[c]	0.86	0.51
8.0	64.3	0.84[c]		0.49
7.5	64.4	0.20[c]		0.16
7.0	65.2	0.090[c]		0.05
10.0	57.1	25[b]		25.1
9.5	57.1	8.2[b]		7.94
9.0	57.5	2.6[b]	2.7	2.57
9.0	57.0	2.9[b]		2.51
8.5	57.2	0.77[c]	0.80	0.81
8.5	57.2	0.82[c]		0.81
10	48.1	11[b]		11.0
9.5	48.1	5.0[b]	4.0	3.46
9.5	48.0	2.9[b]		3.46
9.0	48.0	1.7[c]	1.2	1.10
9.0	48.4	0.76[b]		1.11
8.5	48.0	0.43[c]		0.35
8.0	48.0	0.22[c]		0.11
10.0	38.4	4.2[b]		4.07
9.5	38.2	1.6[c]		1.27
9.0	39.2	0.51[c]		0.42
8.5	39.0	0.20[c]	0.15	0.13
8.5	38.4	0.09[b]		0.13
10.0	29.6	1.4[b]		1.58
9.5	29.2	0.34[b]		0.491
9.0	29.8	0.134[b]		0.159
9.0	29.5	0.203[b]		0.158

[a] Calculated from $k = k_{OH^-}[OH^-]$ where $\log k_{OH^-} = -1.53 \times 10^{-3}(1/T) + 5.12$ and $[OH^-] = 10^{-pOH}$ where pOH = pKw − pH where pKw is given in Table II. [b] Less than 10% loss of volume at 90% hydrolysis. [c] A 10–25% loss of volume at 90% hydrolysis.

The hydrocortisone hemisuccinate, theoretical molecular weight 462, was titrated in 50% ethanol and had a calculated molecular weight of 472, pKa = 5.64, and in 20% ethanol, pKa = 5.10.

CALCULATIONS

The Cannon titrator plots the consumption of standard alkali against time while the solution of hydrocortisone hemisuccinate is maintained at a given pH. Typical curves for the standard alkali consumptions for several pH values at 48° are given in Fig. 1.

The apparent first-order rate constant, k, for the hydrolysis of the hemiester was calculated by the Guggenheim method (4).

The basic equation is

$$\log (\lambda_{(t+\tau)} - \lambda_t) = -kt/2.303 + \text{constant} \quad \text{(Eq. 1)}$$

where k is the apparent first-order rate constant for the hydrolysis of hydrocortisone hemisuccinate at a specific pH, λ_t are the microliters of standard alkali titer consumed, $\lambda_{(t+\tau)}$ are the microliters of standard alkali titer consumed at time $t + \tau$ or τ time units later than time t, and τ is a time interval chosen to permit estimation of the first-order rate constant k in sec.$^{-1}$ without excessively weighting an extrapolated asymptotic value or a value of theoretical alkali consumption. The value τ should exceed the half-life of the reaction.

Typical first-order plots by this Guggenheim method are given in Fig. 2 for several pH values.

The determined rate constants and the pH and temperature of determination are given in Table I.

The rate of alkali consumption, $d[\text{NaOH}]/dt$, can be postulated as equal to the rate of hydrolysis of the hemisuccinate ester, $-d[\text{X}]/dt$, and may be proportional to the products of the concentrations of the hydroxyl ion concentration $[OH^-]$ and the ester concentration [X] so that

$$-d[\text{NaOH}]/dt = -d[\text{X}]/dt = -k[\text{X}] = -k_{OH^-}[OH^-][\text{X}] \quad \text{(Eq. 2)}$$

where at constant pH

$$k = k_{OH^-}[OH^-] \quad \text{(Eq. 3)}$$

and the k values listed in sec.$^{-1}$ in Table I are determined from the slopes of the plots of the Guggenheim Eq. 1 as demonstrated in Fig. 2.

The logarithmic expression derived from Eq. 3 is

$$\log k = \log k_{OH^-} + \log [OH^-] = \log k_{OH^-} - \text{pOH} \quad \text{(Eq. 4)}$$

but

$$\text{pOH} + \text{pH} = \text{pKw} \quad \text{(Eq. 5)}$$

so that

$$\log k = \log k_{OH^-} - \text{pKw} + \text{pH} \quad \text{(Eq. 6)}$$

where the pKw values at various temperatures are listed in Table II from Harned and Owen (5a).

A plot of $\log k$ against pH should be a straight line with an intercept of $\log k_{OH^-} - \text{pKw}$. Such plots are given in Fig. 3. The linearity of the plots is in good agreement with the *a priori* bases of Eq. (6).

The plots of Fig. 3 are fitted to a slope of one and permit the determination of the intercepts, $\log k_{OH^-} - \text{pKw}$, which are given in Table II for the average of the various temperatures studied. The actual temperatures varied among runs by about 0.5° (see Table I) so that the experimental error was actually less than appears from the scatter about the regressions of Fig. 3. Also, in Table I are listed the calculated k values as determined by

$$k = k_{OH^-}[OH^-] = k_{OH^-}[10^{-\text{pKw} + \text{pH}}] \quad \text{(Eq. 7)}$$

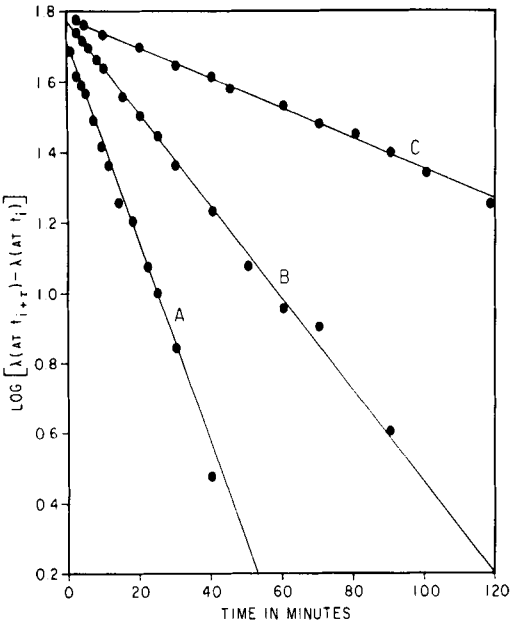


Fig. 2.—Typical first-order plots by the Guggenheim method for the hydrolysis of 0.0165 *M* hydrocortisone hemisuccinate at 48° at constant pH. Curve A is at pH 10.0, curve B is at pH 9.5, and curve C is at pH 9.0. The λ are the numbers of μl. of 2.0 *M* sodium hydroxide consumed at a particular time, *t*. τ is a constant time interval that exceeds the half-life of the reaction.

TABLE II.—ESTIMATES OF THE BIMOLECULAR RATE CONSTANT k_{OH^-} (1/*M*/sec.) FOR THE HYDROXYL ION CATALYZED HYDROLYSIS OF HYDROCORTISONE HEMISUCCINATE

°C.	log k_{OH^-} − pKw[a]	pKw[b]	log k_{OH^-}[c]	k_{OH^-} (1/*M*/sec.)
29	−13.82	13.87	0.05	1.12
38	−13.38	13.60	0.22	1.66
48	−12.96	13.32	0.36	2.29
57	−12.61	13.08	0.47	2.93
65	−12.32	12.89	0.57	3.72

[a] Intercept of the plots, Fig. 3, of the logarithm of the first-order rate constants, *k* (sec.$^{-1}$), at a given temperature, against the observed pH as per log *k* = pH + (log k_{OH^-} − pKw). [b] As given in Harned and Owen (5a). [c] Intercept of plot specified in footnote *a* + pKw.

where the pKw's are given in Table II and the k_{OH^-} is determined for the Arrhenius relation as discussed in the following section. These calculated values show reasonable agreement with the experimental.

Variation of Rate with Temperature.—Arrhenius plots of the logarithm of the apparent first-order rate constant in sec.$^{-1}$, i.e., log *k*, against 1/*T* for various controlled pH values are given in Fig. 4. The slopes of these plots are 4.327 × 10^3 so that the apparent heats of activation, ΔH_a, are 19.8 Kcal./mole as per the expression

$$k = Pe^{-\Delta H_a/RT} \qquad \text{(Eq. 8)}$$

or expressed logarithmically in accordance with the plot of Fig. 4

$$\log k = \log P - (\Delta H_a/2.303R)(1/T) \qquad \text{(Eq. 9)}$$

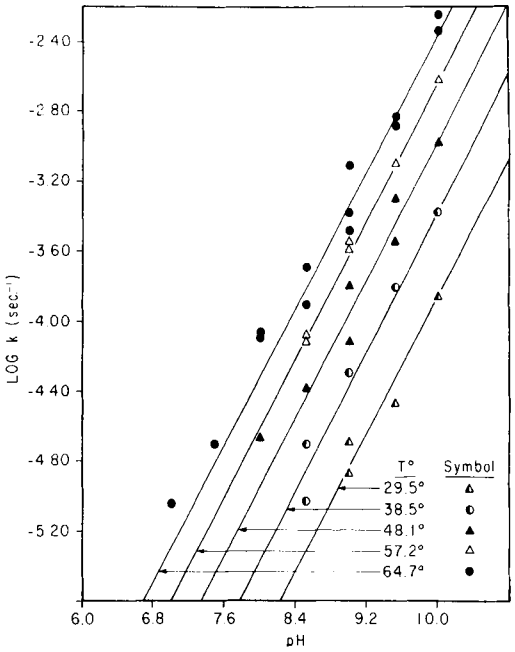


Fig. 3.—Logarithm of apparent first-order rate constants, *k* in sec.$^{-1}$, for the constant pH hydrolysis of 0.0165 *M* hydrocortisone hemisuccinate plotted against pH.

TABLE III.—LOG *P* VALUES FOR THE ARRHENIUS RELATION[a] BETWEEN TEMPERATURE AND THE LOGARITHMS OF THE APPARENT FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF HYDROCORTISONE HEMISUCCINATE AT VARIOUS pH VALUES

pH	log *P*
10.0	11.50
9.5	10.05
9.0	9.53
8.5	9.03
8.0	8.74

[a] $\log k = -\Delta H_a/2.303R(1/T) + \log P$, *k* in sec.$^{-1}$.

where *T* is in absolute degrees. The log *P* values for the various controlled pH values are given in Table III.

Now the apparent first-order rate constant, *k*, is the product of the specific hydroxyl ion rate constant, k_{OH^-}, and the hydroxyl ion concentration as shown in Eq. 3. Each of these factors can be expected to vary with temperature as expressed in Arrhenius relations

$$k_{OH^-} = P_1e^{-\Delta H_1/RT} \qquad \text{(Eq. 10)}$$

$$[OH^-] = Kw/[H^+] = P_2e^{-\Delta H_2/RT}/[H^+] \qquad \text{(Eq. 11)}$$

where ΔH_2 is the heat of ionization of water, 12.8 Kcal./mole at 40° (5b). Thus

$$k = k_{OH^-}[OH^-] = P_1P_2e^{-(\Delta H_1 + \Delta H_2)/RT}/[H^+] \qquad \text{(Eq. 12)}$$

and logarithmically

$$\log k = \log P_1P_2 + \text{pH} - \{(\Delta H_1 + \Delta H_2)/2.303R\}(1/T) \qquad \text{(Eq. 13a)}$$

$$= \log P - \{\Delta H_a/2.303R\}(1/T) \qquad \text{(Eq. 13b)}$$

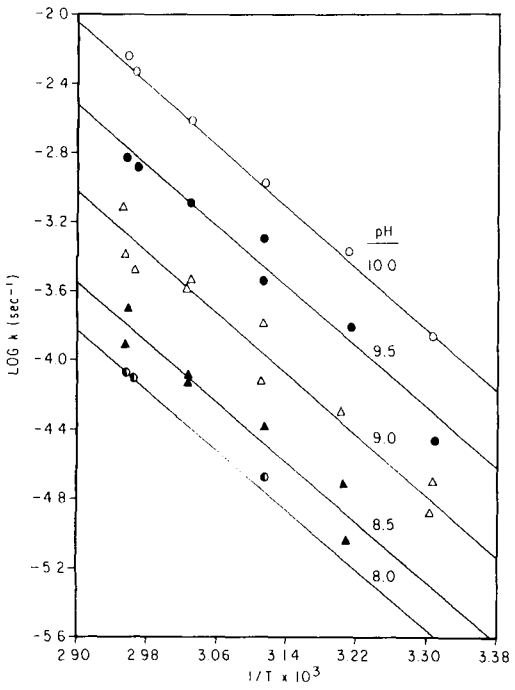


Fig. 4.—Arrhenius plot for the apparent first-order hydrolysis of 0.0165 *M* hydrocortisone hemisuccinate by constant pH titrations.

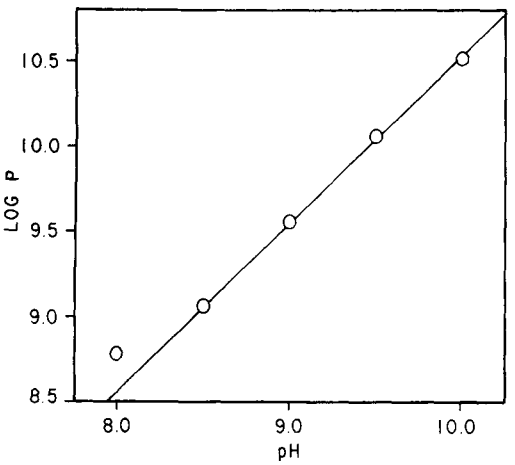


Fig. 5.—Plot of the intercept, log *P*, of the Arrhenius plots, log *k vs.* $1/T$, for the hydrolysis of hydrocortisone hemisuccinate against the pH of hydrolysis.

The intercepts of log *k vs.* $1/T$ in Fig. 4 for a given pH are log P = log P_1P_2 + pH, so that if these intercept values as given in Table III are plotted against pH, the result should be a straight line of unit slope and intercept log P_1P_2. Figure 5 demonstrates the validity of this rationale and permits an estimate of

$$\log P_1P_2 = \log P_1 + \log P_2 = 0.53 \quad \text{(Eq. 14)}$$

A plot of log Kw *vs.* $1/T$ from the data in the literature (5a) gives an apparent ΔH_2 as 12.8 Kcal./mole and log P_2 as −4.61. Thus log P_1 should be 5.14 from Eq. 14. Since ΔH_a for log *k vs.* $1/T$ for all pH values is 19.8 Kcal./mole, $\Delta H_1 = \Delta H_a - \Delta H_2$ should be 7.0 Kcal./mole for the bimolecular rate constant, k_{OH^-}.

The ΔH_1 and log P_1 can be determined directly from the k_{OH^-} values listed in Table II and plotted as per the logarithmic form of Eq. 10 in Fig. 6. The resultant expression is

$$\log k_{OH^-} = -1.53 \times 10^3(1/T) + 5.12 \quad \text{(Eq. 15)}$$

where ΔH_1 = 7.00 Kcal./mole in agreement with the ΔH_1 = 7.0 derived from the relations expressed in the Eqs. 13a and 13b, and log P_1 = 5.12 in agreement with the log P_1 = 5.14 derived from Eq. 14.

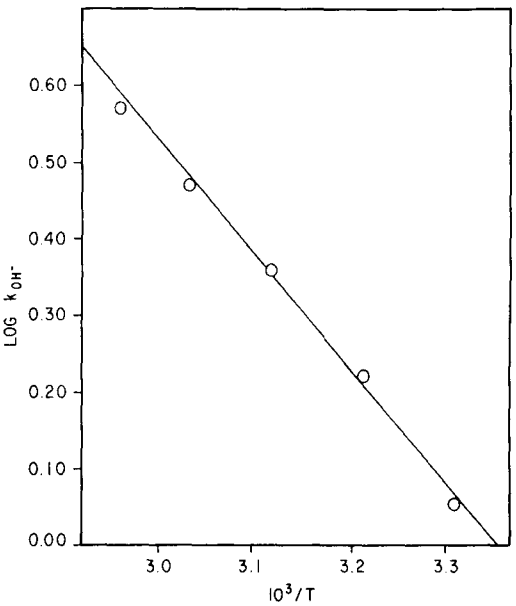


Fig. 6.—Arrhenius plot of the bimolecular rate constant, k_{OH^-} in L./*M*/sec., for the hydroxyl ion catalyzed hydrolysis of hydrocortisone hemisuccinate.

DISCUSSION

The rates of hydrolysis of hydrocortisone hemisuccinate, [X], can be calculated from the following expressions

$$dX/dt = -k[X] \quad \text{(Eq. 16)}$$

or

$$\log X/X_0 = -(k/2.303)t \quad \text{(Eq. 17)}$$

where

$$k = k_{OH^-}[OH^-] \quad \text{(Eq. 18)}$$

and

$$\log k_{OH^-} = -1.53 \times 10^3\,(1/T) + 5.12 \quad \text{(Eq. 19)}$$

and

$$[OH^-] = 10^{-pOH} \quad \text{(Eq. 20)}$$

where

$$pOH = pKw + pH \quad \text{(Eq. 21)}$$

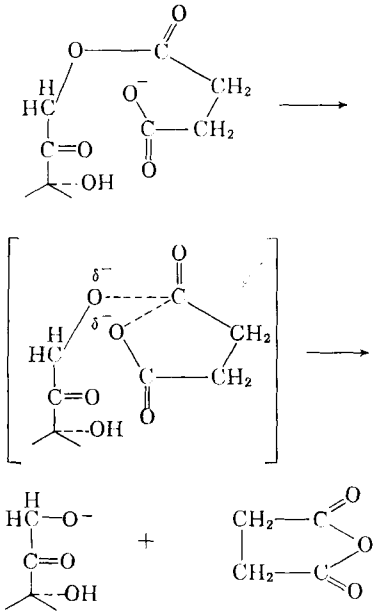
where pH is experimentally observed and pKw = $2.8 \times 10^{-3}(1/T) + 4.61$ (5a).

Rate constants calculated on these premises are compared with the experimental values in Table I and are reasonably consistent. The determination of alkaline hydrolysis rates by measurement of alkali consumption at constant pH in these studies is obviously not as precise as would be liked. The experimental setup was such that solvent evaporation was significant, especially at higher temperatures and for the lower rates at the lower pH values. However, the Guggenheim procedure for the evaluation of first-order reaction rate constants served to minimize the error due to the experimental variation of concentration for prolonged reaction times. Another possible source of error is anomalous pH reading in unbuffered solutions. The internal consistency of the kinetic and thermodynamic equations and the better agreement of the calculated rate with the averages of replicated studies shows that the technique is useful.

Great accuracy is obtained with faster reaction and, in the case of solutions of low ionic concentrations, at higher pH values. The obtained data describe the hydrolysis of hydrocortisone hemisuccinate as being specifically catalyzed by hydroxyl ion above a pH of 8.

Below a pH of 8, the apparent rates of hemisuccinate hydrolysis may be greater than the extrapolation of the specific hydroxyl ion catalysis rate expressions would predict. The values that can be calculated on the basis of these expressions should only be considered as the minimum estimate of the rates at pH values below 8.

Certain chemical phenomena that may produce rates below pH 8 that exceed prediction may be (*a*) the increase in the relative importance of spontaneous hydrolysis or intramolecular attack of the anion on the ester carbonyl carbon (6–10)



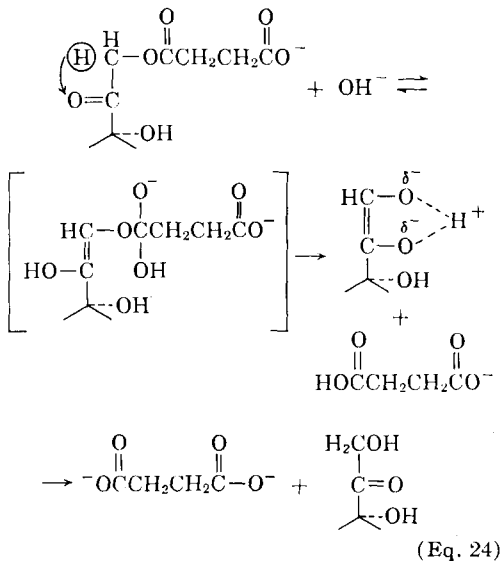
(Eq. 22)

or (*b*) a significant contribution of hydronium ion catalysis to hemisuccinate hydrolysis at pH values below 8 since this would involve a reaction accelerated by the attraction of opposite charges

$$H_3O^+ + R{-}OCOCH_2CH_2O^- \rightarrow R{-}OH + HOOCCH_2CH_2COOH \quad \text{(Eq. 23)}$$

The heat of activation for the specific hydroxyl ion catalysis of hydrocortisone hemisuccinate is 7.0 Kcal./mole and is significantly less than that found for the alkaline hydrolysis of hemisuccinic acid ester of secondary alcohols, i.e., 9–14 Kcal./mole (11–13). The rate constants for hydrocortisone hemisuccinate hydrolysis are also much higher than for other succinate esters. For example, at 40° the rate constant is *ca.* 200 times greater than that for the hydrolysis of cyclohexanol hemisuccinate (12).

These phenomena imply that the structure of the steroid side chain is such that it promotes 21 ester hydrolysis over and above possible repulsion of the attacking hydroxyl ion by a negative charge on the molecule. A possible explanation is that a leaving group is stabilized by resonance, a group such as an enediol anion



(Eq. 24)

The major conclusion is that a 21-steroid ester is highly susceptible to alkaline hydrolysis by virtue of the unique structure of the steroid side chain.[2]

The stability of hydrocortisone hemisuccinate can be predicted at 25° by application of Eq. 15 where k_{OH^-} at 25° = 1.0 and pKw = 14. The predictions of half-life are given in Table IV.

TABLE IV.—PREDICTED HALF-LIVES OF HYDROCORTISONE HEMISUCCINATE AT VARIOUS pH VALUES AT 25°

pH	$t_{1/2}$
7	80 days
8	8 days
9	19 hours
10	1.9 hours

[2] A possible alternative mechanism to postulate is one where the 17α-hydroxyl group is involved as an assisting group. Dr. S. Morris Kupchan of The University of Wisconsin suggests that this is consistent with his studies.

Recent studies on blood levels from parenterally administered hydrocortisone-21-hemisuccinate (14, 15) have shown that the steroid administered in this form is quickly available to the tissues. It was also shown that levels of free steroid after injections of the 21-phosphate ester were higher than those obtained after the 21-hemisuccinate esters were injected. Since comparative studies on biological activities are lacking, no conclusion as to medical significance could be drawn (15).

However, it was speculated (14) that a portion of the succinate ester was metabolically transformed without prior hydrolysis. Contrarily, no significant differences were observed between the phosphate and succinate esters studied in terms of percentage of injected steroid excreted in the urine.

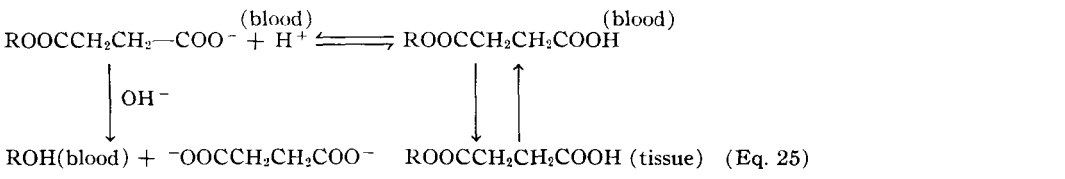
A reasonable explanation for the blood level difference in free steroid is the greater deposition of hydrocortisone hemisuccinate in the tissues over that of the phosphate. Hydrocortisone hemisuccinate has a pKa *ca.* 5.0, and at the neutral pH of the blood, the sodium salt is in equilibrium with the free uncharged hemiester. In general, uncharged molecules exhibit higher lipoidal or fat solubility than charged molecules and penetrate cell membranes more rapidly

On the basis of Marcus' equation relating hydrolysis rate with pH and temperature (18), the apparent first-order rate constant for the production of free steroid from the phosphate ester is predicted as 2.5 $\times 10^{-4}$ hr.$^{-1}$ at 38° and pH 7.0. On the basis of the experimental data in this paper, and by use of Eq. 15 with pKw = 13.60 at 38° so that $[OH^-] = 2.51 \times 10^{-7}$ at pH 7, the apparent first-order rate constant for the hydrolysis of hemisuccinate ester is predicted to be 1.3 $\times 10^{-3}$ hr.$^{-1}$ at 38° and pH 7.0. It follows that, on the basis of solvolysis alone, free steroid should be produced from the succinate ester *ca.* five times faster than from the phosphate ester.

This fact is inconsistent with an assumption that 21-phosphate esters more readily produce free steroid in the blood than the 21-hemisuccinate esters by solvolytic mechanisms. This fact strongly validates the model proposed in Eq. 25.

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(Eq. 25)

This fact has been well discussed in the literature (16, 17).

The doubly charged steroid phosphate is the major form of hydrocortisone phosphate at physiological pH values and has two pKa's, 6.2 and *ca.* 2 (18). At neutral pH the doubly charged phosphate anion is in equilibrium with the singly charged phosphate anion

$$R—OPO_2^{=} + H^+ \rightleftharpoons R—OPO_2H^- \quad \text{(Eq. 26)}$$

Thus, uncharged phosphate ester would be available for diffusion into lipid-like membranes only if the pH were decreased to *ca.* 4 and this is not physiologically feasible.

It follows, therefore, that increased tissue permeability of the hemisuccinate ester over the phosphate ester could account for the lower blood levels of free alcohol from the former.

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Prediction of Stability in Pharmaceutical Preparations XI

Increased Stability of Soluble Steroid Hemiesters by Steric Hindrance

By EDWARD R. GARRETT† and MAX E. ROYER

Since alkyl substitution on the mono-esterified dicarboxylic acid of 21-steroid hemiesters decreases the rate of specific hydroxyl ion catalyzed hydrolysis, the kinetics of aqueous hydrolysis of prednisolone and 6α-methylprednisolone hemi-β,β′-dimethylglutarates have been studied by constant pH titration. The effect of steroid 6α-substitution on rate of 21-ester hydrolysis has been indicated. The stabilities of sodium salts of such esters at various pH values between 8 and 10 and at various temperatures have been predicted. It has been shown that steric hindrance due to alkyl substitution on the monoesterified dicarboxylic acid increases stability by more than tenfold over the previously reported stability of the nonhindered hydrocortisone hemisuccinate. A more precise procedure for constant pH titration is described.

THE PHARMACEUTICAL utility of a steroid has been enhanced by formation of a soluble derivative that still possessed biological potency and stability as the derivative. The criteria of solubility and biological potency have been well fulfilled by the sodium salt of the hemisuccinate ester of hydrocortisone (1–3). It has been shown that the stability of 21-steroid hemiesters is not significantly affected by increasing the chain length of the monoesterified dicarboxylic acid (4). Resistance to specific hydroxyl ion catalysis is promoted, however, by alkyl substitution on the dicarboxylic acid in accordance with Newman's rule of "six" (5, 6).

This paper reports on the detailed kinetic studies of the specific hydroxyl ion catalyzed hydrolysis of such sterically hindered esters, the hemi-β,β′-dimethylglutarate esters of prednisolone and 6α-methylprednisolone in aqueous media.

EXPERIMENTAL

The procedure for the constant pH titrations of the sodium salt of the hemi-β,β′-dimethylglutarate ester of prednisolone (Δ¹-hydrocortisone) was similar to that reported previously (4, 7). However, the experimental techniques for the constant pH titrations of the sodium salt of the hemi-β,β′-dimethylglutarate ester of 6α-methylprednisolone were considerably improved. The material was weighed and placed in a beaker fitted with a close-fitting rubber insert through which the single Beckman compound electrode extended to the bottom of the beaker. A metal clamp was also used to seal this rubber cap around the beaker. A needle was placed through the rubber insert and tubing led to another needle sealed in the electrode side orifice so that the pressure atop the electrode salt solution was the same as that within the beaker. The beaker containing the weighed material and a magnetic bar was submerged in the constant temperature bath atop a magnetic stirrer in a watertight can with a conduit periscoped above the water to permit egress for the insulated electric cord.

Fifteen milliliters of nitrogen-purged water, previously equilibrated in the temperature bath, was added by means of a syringe and needle thrust through the rubber cap. An additional needle had been thrust through the insert to act as a vent.

The resultant solution was *ca.* 0.004 *M* in hemiester. Both of these needles were removed and a 25-gauge 2-in. needle, bent at a right angle, was inserted. This was for the titrant delivery from a gas-tight 1-ml. "microliter" syringe.[1] The rubber cap bulged slightly from the internal vapor pressure but was definitely vaportight.

The Cannon difunctional titrator had been standardized previously as reported (4, 7).

CALCULATIONS AND RESULTS

Typical plots of the consumption of standard sodium hydroxide by hemi-β,β′-dimethylglutarate esters of a steroid alcohol are given in Fig. 1. These plots are for the hydrolysis of the 6α-methylprednisolone ester at 70.0° at several pH values and are given as chart divisions against time. Each chart division, *CD*, represents 0.008052 meq. of sodium hydroxide added as 1.0175 *M*. In some instances, as at pH 10.0, the possible rate of addition of alkali was slower than the consumption so that the only valid part of the curve is when the desired constant pH was attained and maintained.

The dashed lines in Fig. 1 represent the tangents to the curves at the origin and the apparent first-order rate constants, *k*, at the constant pH values can be calculated from

$$k(\text{sec.}^{-1}) = (N \times m)/(m' \times M) = (CD \times 0.008052)/(m' \times M) \quad \text{(Eq. 1)}$$

where *m* is the number of ml. of *N* normal sodium hydroxide consumed per second at the constant pH

Received August 4, 1961, from the Research Division, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication September 21, 1961.
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[1] Syringe No. 1001, Hamilton Co., Whittier, Calif.

TABLE I.—RATE CONSTANTS (10^5 k in sec.$^{-1}$) FOR THE APPARENT FIRST-ORDER HYDROLYSIS OF 0.004 M STEROID HEMIESTERS OF β,β'-DIMETHYLGLUTARATE AT VARIOUS TEMPERATURES AND pH VALUES BY CONSTANT pH TITRATION OF 15 ML. WITH 1.0 M SODIUM HYDROXIDE

	6α-Methylprednisolone			Prednisolone		
pH	70.0°	60.0°	50.0°	67.5°	57.5°	50.5°
10.50	...	...	...	...	...	97.3,[a] 98.3[b]
10.00	126,[a] 116[b]	27.8,[a] 25.3[b] 38.8,[a,c] 28.5[b,c]	9.93,[a] 9.43[b]	91.6,[a] 94.4	37.8,[a] 38.7[b]	24.4,[a] 25.5[b]
9.50	37.5,[a] 34.5[b]	10.5,[a] 10.1[b] 8.86,[a,c] 8.69[b,c]	3.68,[a] 2.88[b]	30.7,[a] 24.2[b]	14.9,[a] 13.5[b]	6.49,[a] 4.96[b] 6.72,[a,c] 6.20[b,c]
9.00	10.1,[a] 8.58[b]	2.81,[c] 2.78[b]	0.878,[a] 0.863[b]	17.0,[a] 13.9[b]	...	1.96,[a] 1.79[b]
8.75	4.59,[a] 3.40[b]	...	...	...	...	...
8.54	...	...	...	...	2.10[a]	...
8.50	3.28,[a] 3.40[b]	...	...	3.15,[a] 3.33[b]	...	...

[a] Calculated from slope of plot of first-order expression ($\log \lambda_\infty - \lambda$) = $-kt/2.303 + \log \lambda_\infty$ where λ_∞ is the theoretical volume of standard titer consumed on complete saponification and λ is the amount consumed at time, t. [b] Calculated from initial zero-order slope, m, of ml. of N normal titer consumed per second by m' ml. of M molar steroid hemiester, $k = (N \times m)/(m' \times M)$. [c] A different study than the one above it.

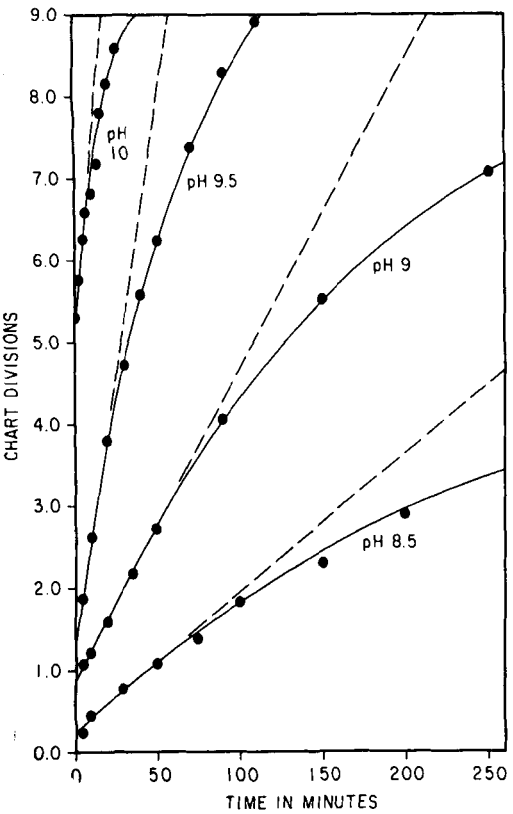


Fig. 1.—Typical plots of consumption of 1.000 M sodium hydroxide by 15 ml. of 0.004 M 6α-methylprednisolone hemi-β,β'-dimethylglutarate at constant pH and at 70.0° as a function of time. Each chart division represents 0.008052 meq. sodium hydroxide. The dashed lines are estimates of the initial rate of sodium hydroxide addition. At pH 10, the titer addition did not initially keep up with speed of reaction.

by m' ml. of M molar hemiester, or where CD is the number of chart divisions where each chart division represents 0.008052 meq. sodium hydroxide added.

The first-order rate constants, k in sec.$^{-1}$, derived from these tangents at the origin to the plots of alkali consumption with time are given in Table I for both 6α-methylprednisolone and prednisolone hemi-β,β'-dimethylglutarate.

Typical first-order plots are given in Fig. 2 as based on the expression

$$\log (CD_\infty - CD) = -kt/2.303 + \log CD_\infty \quad \text{(Eq. 2)}$$

where CD_∞ is the number of chart divisions which should be traveled at theoretically complete saponification of the *ca.* 0.004 M ester in the 15 ml. of water.

The apparent first-order rate constants, k in sec.$^{-1}$, estimated from the slopes of such plots are also given in Table I. The fine consistency of the rates determined by this method and calculated from the tangent to the origin is gratifying.

Plots of the logarithm of the rate constants against pH for the 6α-methylprednisolone and prednisolone hemiesters are given in Figs. 3 and 4, respectively.

These plots are characterized by the equation

$$k = k_{OH^-}[OH^-] \quad \text{(Eq. 3)}$$

or in logarithmic form

$$\log k = \log k_{OH^-} - \text{pOH} = \log k_{OH^-} + \text{pH} - \text{pKw} \quad \text{(Eq. 4)}$$

The intercepts of the plots of Figs. 3 and 4 are $\log k_{OH^-} - \text{pKw}$ and are given in Table II. The $\log k_{OH^-}$ can thus be calculated from the knowledge of the pKw at the given temperature as listed in the literature (8).

The Arrhenius plots of $\log k_{OH^-}$ against the reciprocal of the absolute temperature, $1/T$, are given in Fig. 5 as per the expression

$$\log k_{OH^-} = -S/T + \log P = \Delta H_a/2.303\ RT + \log P \quad \text{(Eq. 5)}$$

where for 6α-methylprednisolone hemi-β,β'-dimethylglutarate S = 2,995, $\log P$ = 8.506, and ΔH_a = 13.7 Kcal./mole and for prednisolone hemi-β,β'-dimethylglutarate S = 2,422, $\log P$ = 7.050, and ΔH_a = 11.1 Kcal./mole.

Values of $\log k_{OH^-}$ at 25, 30, and 40° can be calculated for both hemiesters from Eq. 5 with proper substitution of the derived parameters and they are given in Table II. The apparent first-order rate constants can be calculated from the derived values of Table II by insertion into Eq. 4 and are given for various pH values in Table III.

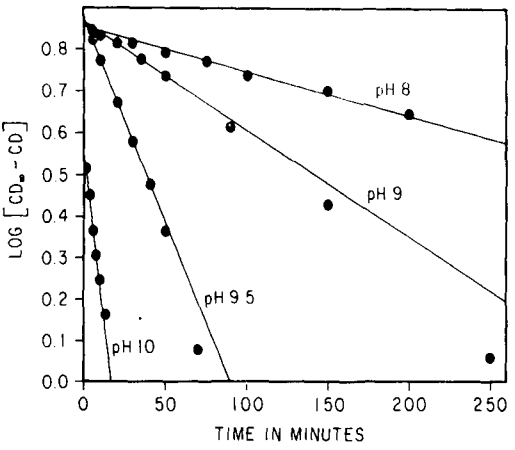


Fig. 2.—Typical first-order plots of hydrolysis of 6α-methylprednisolone hemi-β,β′-dimethylglutarate at constant pH and at 70°. Each chart division, *CD*, represents 0.008052 meq. sodium hydroxide added as 1 *M* to 15 ml. of *ca.* 0.004 *M* steroid hemiester. CD_∞ is the theoretical value at complete saponification.

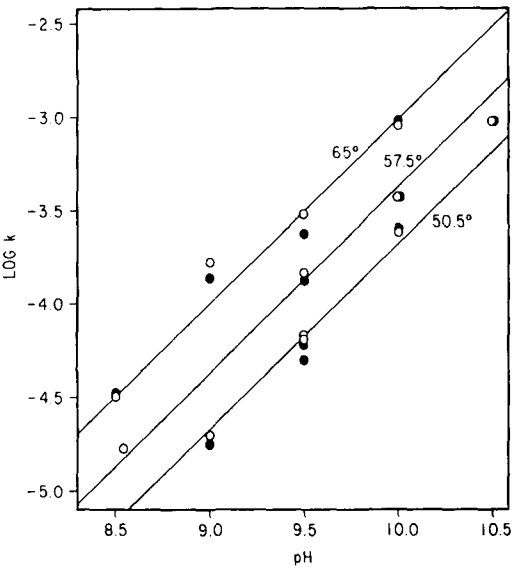


Fig. 3.—Logarithm of the apparent first-order rate constants, *k* in sec.$^{-1}$, for the hydrolysis of 6α-methylprednisolone hemi-β,β′-dimethylglutarate as a function of pH. The open circles represent *k* values derived from first-order plots; the solid circles represent *k* values derived from the initial zero-order rate of alkali consumption; the half-open circles are coincident values.

The half-life, $t_{1/2}$ in days, is calculated from

$$t_{1/2}(da) = 8.00 \times 10^{-6}/k(\text{sec.}^{-1}) \quad \text{(Eq. 6)}$$

The fraction, *f*, of nonhydrolyzed ester remaining at one year at the given temperature and pH and given in Table III is calculated from

$$\log f = -1.367 \times 10^{7} k(\text{sec.}^{-1}) \quad \text{(Eq. 7)}$$

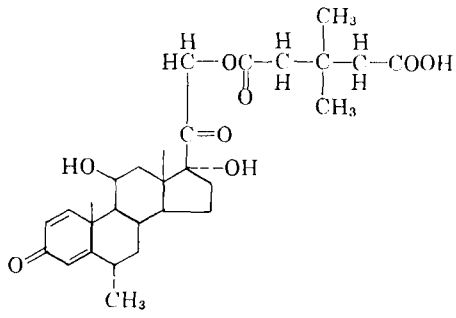
TABLE II.—ESTIMATES OF THE BIMOLECULAR RATE CONSTANT k_{OH^-} (1/*M*/sec.) FOR THE HYDROXYL ION CATALYZED HYDROLYSIS OF HEMIESTERS OF β,β′-DIMETHYLGLUTARATE

	°C.	log k_{OH^-} − pKw[a]	pKw[b]	log k_{OH^-}	(1/*M*/sec.) k_{OH^-}
6α-Methyl-prednisolone	70.0	−13.00	12.76	−0.24	0.58
	60.0	−13.50	13.02	−0.48	0.32
	50.0	−14.03	13.26	−0.77	0.17
	40.0[c]	−14.59	13.53	−1.06	0.087
	30.0[c]	−15.22	13.84	−1.38	0.042
	25.0[c]	−15.55	14.00	−1.55	0.028
Prednisolone	65.0	−13.01	12.89	−0.12	0.76
	57.5	−13.37	13.07	−0.30	0.50
	50.5	−13.68	13.25	−0.43	0.37
	40.0[c]	−14.22	13.53	−0.69	0.20
	30.0[c]	−14.78	13.84	−0.94	0.12
	25.0[c]	−15.08	14.00	−1.08	0.083

[a] Intercept of plot of logarithm of apparent first-order rate constant, *k* in sec.$^{-1}$, against pH by the expression log k = log k_{OH^-} − pKw + pH. [b] As determined from ref. 8. [c] The listed values for these temperatures were calculated from the derived Arrhenius' expression log $k_{OH^-} = -S/T + \log P$ where *S* and *P* for the 6α-methylprednisolone hemiester are $2.995 \cdot 10^3$ and 8.506, respectively, and for the prednisolone hemiester are $2.442 \cdot 10^3$ and 7.050, respectively. From these data and the pKw, the other values can be derived.

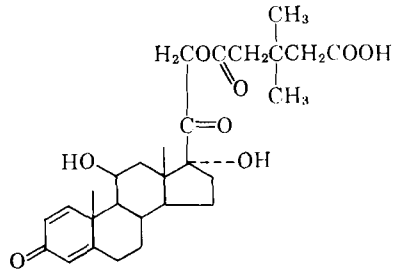
DISCUSSION

The specific hydroxyl ion catalyzed hydrolysis of 6α-methylprednisolone hemi-β,β′-dimethylglutarate (I) would be expected to have rates similar to the



I

specific hydroxyl ion catalyzed hydrolysis of prednisolone hemi-β,β′-dimethylglutarate (II) since the



II

former compound differs from the latter by only an α-methyl at the 6 position. Surprisingly, the latter compound hydrolyzes faster by an order of magnitude greater than two. Although the experimental conditions were not as elegant for the constant pH hydrolyses of the prednisolone compound as for the 6α-methylprednisolone since the improvements were subsequently introduced, the difference in magnitude of rates is considered significant.

TABLE III.—PREDICTED HYDROLYSIS RATES[a] OF STEROID ESTERS OF HEMI β,β'-DIMETHYLGLUTARATE[b]

pH	25° 10^8k (sec.$^{-1}$)[a]	25° $t_{1/2(da)}$[c]	25° f[d]	30° 10^8k (sec.$^{-1}$)[a]	30° $t_{1/2(da)}$[c]	30° f	40° 10^7k (sec.$^{-1}$)[a]	40° $t_{1/2(da)}$[c]	40° f[d]
				6α-Methylprednisolone					
10	282	2.84	...	602	1.50	...	257	0.311	...
9	28.2	28.4	0.00	60.2	15.0	0.00	25.7	3.11	...
8	2.82	284	0.41	6.02	150	0.15	2.57	31.1	...
7	0.282	2,840	0.92	0.602	1,500	0.83	0.257	311	0.45
				Prednisolone					
10	831	0.961	...	1,660	0.482	...	602	0.133	...
9	83.1	9.61	...	166	4.82	...	60.2	1.33	...
8	8.31	96.1	0.07	16.6	48.2	0.005	6.02	13.3	...
7	0.831	961	0.77	1.66	482	0.59	0.602	133	0.15

[a] Log k_{OH^-} was calculated from log $k_{OH^-} = -2.995 \cdot 10^3(1/T) + 8.506$ for 6α-methylprednisolone hemiester, log $k_{OH^-} = -2.422 \cdot 10^3(1/T) + 7.050$ for prednisolone hemiester where T = °C. + 273°. Subsequently, k for a given pH and temperature was estimated by log k = log k_{OH^-} − pKw + pH, where pKw for a given temperature is given in Table II from the literature (8). [b] The ester of 6α-methylprednisolone hydrolyzes 0.029× as fast as hydrocortisone hemisuccinate at 25°, 0.036× as fast at 30°, and 0.051× as fast at 40°. The ester of prednisolone hydrolyzes 0.085× as fast as hydrocortisone hemisuccinate at 25°, 0.10× as fast at 30°, and 0.12× as fast at 40°. [c] Half-life in days at the given pH, $t_{1/2(da)} = 8 \times 10^{-6}/k$(in sec.$^{-1}$). [d] The fraction of nonhydrolyzed ester remaining at one year at the given temperature and pH.

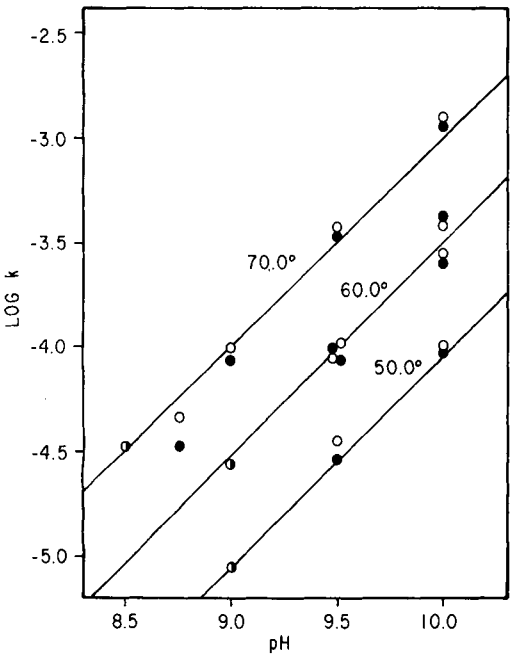


Fig. 4.—Logarithm of the apparent first-order rate constants, k in sec.$^{-1}$, for the hydrolysis of prednisolone hemi-β,β'-dimethylglutarate as a function of pH. The open circles represent k values derived from first-order plots; the solid circles represent k values derived from the initial zero-order rate of alkali consumption.

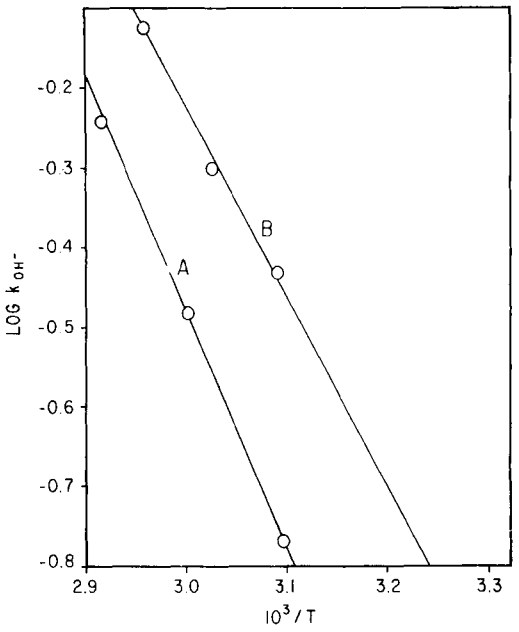
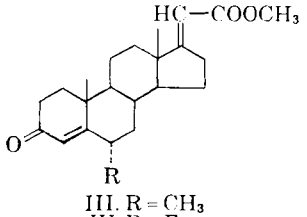


Fig. 5.—Arrhenius plot of the bimolecular rate constant, k_{OH^-}(1/M/sec.) for the hydroxyl ion catalyzed hydrolysis of steroid hemiesters of β,β'-dimethylglutarate. Curve A is for 6α-methylprednisolone; curve B is for prednisolone.

It is also significant that the coulometric bromination of methyl-6α-methyl-4,17(20)-[*cis*]pregnadien-21-oate (III) proceeds exceedingly fast whereas the coulometric bromination at fifty times the bromine concentrations as used for III is very much slower for methyl-6α-fluoro-4,17(20)-[*cis*]-pregnadien-21-oate (IV) (9).

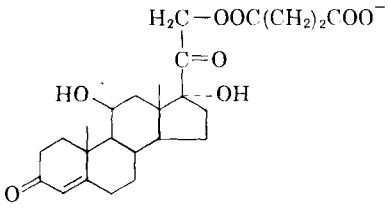


III. R = CH_3
IV. R = F

In addition, it has been shown by derivative ultraviolet spectroscopy that a definitive interaction exists between the 3-keto-Δ^4 system of the A ring modified by a 6α-methyl substitution and an α-hydroxyl substituent at the 17 position (10).

The hydrolysis studies were conducted down to a pH of 8.5 and only specific hydroxyl ion catalysis was indicated. This does not permit rejection of intramolecularly abetted "spontaneous" hydrolysis or, its kinetic equivalent, specific hydroxyl ion catalyzed hydrolysis of the undissociated hemiester

which would make a significant contribution to the total hydrolysis rate at lower pH values, specifically in the neutral pH region 4–7 as a function of pKa. This phenomenon had been indicated previously for hydrocortisone hemisuccinate(V) (4).



V

Thus the predicted stabilities (Table III) of the β,β′-dimethylglutarate hemiesters of these steroids at pH values closer to neutrality are the most optimistic estimates. Intramolecular catalysis, if present, would tend to increase the hydrolysis rate at neutral pH values over that predicted from specific hydroxyl ion catalysis alone.

As was expected, stability to specific hydroxyl ion catalyzed hydrolysis is greatly enhanced by alkyl substitution over that for the hemisuccinate or other straight chain aliphatic hemiesters (4, 7) and is in accordance with Newman's rule (5, 6). It is interesting to note, however, that if intramolecular catalyzed hydrolysis is present, and significantly contributes in the neutral pH region 4–7, it would be predicted to be greater in the case of the alkyl substituted esters than for the nonalkylated hemiesters of dicarboxylic acids such as the straight chain glutaric or succinic acids (11).

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Solubilization in Nonpolar Solvents

Influence of the Chain Length of Solvent on the Solubilization of Water by Dioctyl Sodium Sulfosuccinate

By W. I. HIGUCHI and JAGDISH MISRA

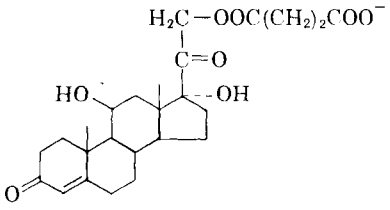
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THE PHENOMENON of micellar solubilization in aqueous systems has recieved considerable attention, this subject having recently been covered extensively in a book by McBain and Hutchinson (1). The applications of the principles and concepts of solubilization in aqueous media to pharmacy have been demonstrated (2). The drug activity, release characteristics, and stability (3, 4) may be improved by a suitable choice and amount of these solubilizing agents.

There appears to have been little or no study of micellar solubilization of drugs in nonaqueous systems. It is not implied by this statement that the practice of pharmacy has not involved the use of this type of solubilization. However, the mechanisms of incorporation of water and highly polar drugs in hydrophobic vehicles by agents have not received much consideration. One should know whether the formulation is

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Supported by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
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truly physically stable thermodynamically or whether the system is an emulsion or a suspension capable of physical degradation. The questions of how much bound water and drug are there and what are their states of binding should be of concern. The results of an investigation along these lines would be helpful in the understanding and in the improvement of oil base formulations, e.g., ointments, lotions, oil injections, etc.

It was decided that a water-surfactant-hydrocarbon compatibility study would be a most worthwhile starting point. Specifically, the aims were to show how much water might be incorporated into an oil with the help of a surfactant and how available such water might be for drug solubilization. Results of this investigation are reported in this communication.

EXPERIMENTAL

General Considerations.—The surfactant used in these studies was dioctyl sodium sulfosuccinate (di-2-ethylhexyl sodium sulfosuccinate).[1] It was selected because some of its micellar characteristics in nonaqueous solvents had been studied by others (5–7). The oils used for this work were some of the normal aliphatic hydrocarbons from heptane to octadecane. These were chosen because preliminary studies had indicated a rather unusual chain length-solubilization limit relationship, *viz.*, that in shorter chain solvents a great deal more water could be solubilized by the same amount of AOT.

Materials.—In preliminary studies "100% pure" AOT was used after removing the methanol-insoluble fraction. However, the end points (turbidity point) in the water solubilization experiments were not reproducible[2] with this purification procedure despite the fact that the carbon, hydrogen, and sulfur analyses indicated better than 99% purity in these samples. Therefore, the following procedure was followed which is a modification of that reported by Williams, Woodberry, and Dixon (8).

About 400 Gm. of AOT was dissolved in 800 ml. of methanol. The insoluble solids were centrifuged and the supernatant removed by decantation and cooled to 3°. To this solution was added about 1.4 L. of water cooled to 3°. The resulting precipitate was centrifuged out at 5° and discarded. Then 0.2 L. more of water at 3° was added to the solution and about 100 to 150 Gm. of the resulting precipitate was recovered by, again, centrifugation. The material was dried under vacuum at 75° to 85° for 2 to 3 days until further drying showed no loss in weight of the material. Samples prepared in this manner always gave the same mole-mole (water-to-surfactant) ratio to within about ±2%.

All of the hydrocarbons used in this work were Eastman practical grade. These were dried and redistilled. Essential purity in all cases was established by melting point determinations. Doubly distilled water was used.

[1] Marketed as Aerosol OT by American Cyanamid Co.
[2] Variations in the results were as great as 30%.

Procedure for Solubilization Limit Study.—To determine the amount of water that could be solubilized before the system became two-phase the following procedure was adopted. Water was added to about 30 ml. of a magnetically stirred AOT-hydrocarbon solution in water-jacketed 250-ml. flasks until turbidity and two-phase formation occurred. In all cases the end point was sharp (within 1% of added water) and reversible. At the end point the systems were initially turbid, but after a few minutes, two separate, relatively nonviscous, layers formed which cleared after a few more minutes of standing. The smaller of the two phase volumes at the end point was always much greater than the amount that could be explained on the basis of only pure water coming out.

Procedure for Vapor Pressure of Water Determinations.—A rapid relative method was devised for determining the vapor pressures, and hence the thermodynamic activities, of the solubilized water. The isopiestic principle was utilized in a novel fashion.

The water vapor in the solubilized system was exposed to a salt solution (sodium chloride or calcium chloride) in a weighing bottle suspended from a glass hook attached to the male ground joint of the flask. The solubilized system was stirred magnetically from the outside, as in the solubilization limit studies. The vapor pressure of the water in salt solution was accurately known from standard tables (9). If after several hours the weighing bottle had gained or lost weight, a salt solution of different water activity was substituted. Generally after three such trials it was found that the water activity in the solubilized system could be matched to within about 0.01 unit of activity (activity = unity for pure water) with these "indicator" salt solutions. Thus, within a day or so a series of vapor pressure determinations was completed. Preliminary tests had shown that the usual procedure of equilibrating the solubilized system with an originally unmatched salt solution would have taken weeks.

RESULTS

Solubilization Limit Determinations.—The results of the mole water per mole AOT solubilizable in a single phase state at 30° are given in Fig. 1 as a function of the AOT concentration (w/w). The AOT concentration refers to the AOT-hydrocarbon solution prior to the addition of water. In Fig. 2 the corresponding results at different temperatures are given for octane, dodecane, and hexadecane.

As already mentioned, the precision of the end point determination for a given run was better than 1% but the reproducibility of a point for different AOT starting materials was about ±2%. Therefore, in order to minimize the effects of these sample variations upon both the temperature and the AOT concentration dependencies, the same stock AOT sample was used for the results given in Figs. 1 and 2.

The most interesting matter is the extreme sensitivity of the turbidity point to the chain length of the solvent. The behavior is somewhat surprising in view of the negligible chemical differences in the solvents.

Even for the heavy hydrocarbons, hexadecane and

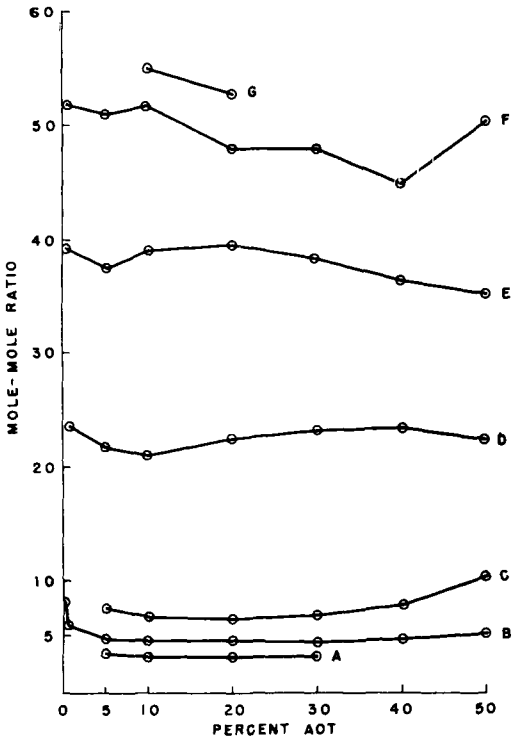


Fig. 1.—The solubilization limit for water in AOT-hydrocarbon systems at 30°. The ordinate is maximum moles of water solubilized per mole AOT. The abscissa is the per cent AOT in hydrocarbon solution prior to addition of water. A, octadecane; B, hexadecane; C, tetradecane; D, dodecane; E, decane; F, octane; and G, heptane.

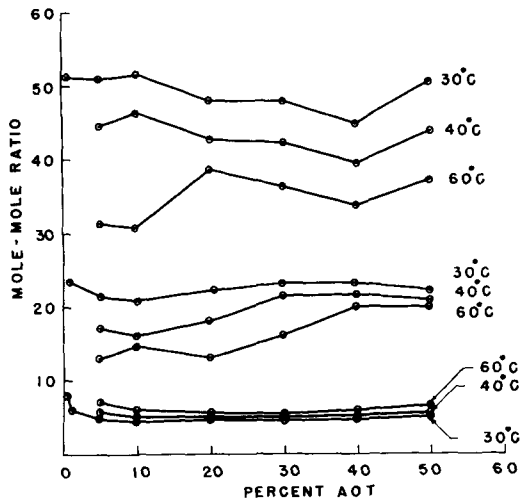


Fig. 2.—The solubilization limit for water in AOT-hydrocarbon systems at different temperatures.

octadecane, four to five moles of water per mole AOT can be sustained in a single phase system. Thus, for the 50% AOT case, it would mean that even here as much as 10% water may be incorporated in a clear preparation.

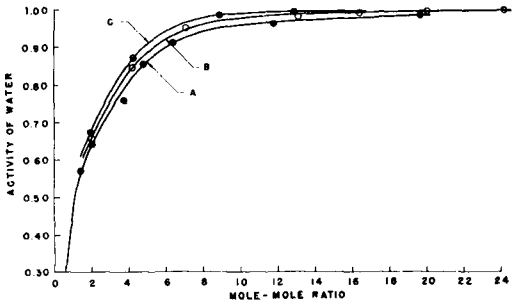


Fig. 3.—The activity of water in AOT-hydrocarbon-water systems as function of mole water per mole AOT solubilized in initially 10% AOT-hydrocarbon solution. A, Hexadecane; B, dodecane; C, octane.

Activity of Water Determinations.—In Fig. 3 the results of the determination of the activity of water (pure water = unit activity) are given for the octane, dodecane, and hexadecane systems at 30° and 10% AOT (in original hydrocarbon solution). The similarity of all three curves suggests that the state of binding of water in the three cases may not be too different, even when compared beyond the hexadecane turbidity point (~5 mole-mole ratio). This at first does appear surprising in view of the great differences in these systems with respect to their solubilization limit behavior.

In the data range reported in Fig. 3 for the octane system the possible condensation of octane in the weighing bottle to give erroneous values was discounted. However, a few activity determinations for the octane system beyond the turbidity point are not reported because there was fogging observed both on the weighing bottle walls and the walls of the flask. The condensing material appeared to be water, and the weighing bottle picked up weight even when pure water was placed in it. These observations suggest that water had just exceeded unit activity at the turbidity point for the octane system.[3]

Because of the fact that for hexadecane, and possibly for dodecane,[4] the turbidity point occurred below unit water activity, the solubilization limits as determined here cannot correspond to pure water coming out as the second phase, at least for hexadecane. Therefore, even up to about 20 moles water per mole AOT in the hexadecane case (see Fig. 3), water seems to be in the micellized state even though beyond 5 moles of water the system is no longer a single phase.

DISCUSSION

Mechanism of Solubilization and Solubilization Limit Behavior.—The ultracentrifuge and viscosity studies on the dodecane-AOT-water system by Mathews and Hirschhorn (5) have shown that in this system the water is bound in the interior of spherical monodispersed micelles. Studies by Aebi and Wiebush (7), also on the dodecane system, sug-

[3] The water in a water-in-oil emulsion may possess activity slightly greater than bulk water because of the surface-free energy of the droplets.

[4] As stated, the activity determinations were good to about 1%. For the dodecane system the turbidity occurred at a water activity of 0.99 ± 0.01, but no fogging of flask or bottle walls was observed at this point.

gest that about one or two moles of water per mole AOT in these micelles are very tightly bound by the polar heads of the AOT while the remainder of the water is "free." This free water is able to dissolve sodium chloride to essentially the same extent as bulk water, except for the common ion effect due to the Na^+ contribution from the AOT itself. In view of the activity data given in Fig. 3, this picture for the state of dispersion of the system should be appropriate for both octane and hexadecane, and therefore for probably all hydrocarbons studied here.

Why then are there such vast differences in the solubilization limits? An unambiguous answer to this question would require more work on this problem. The situation is complex to the extent that a single simple mechanism will not explain all of the facts. In the following paragraphs a tentative, reasonable picture consistent with the main observations is presented.

If it is first postulated that the turbidity point corresponds to the aggregation of intact swollen micelles and that the aggregation tendency is inversely related to the degree of solvation of the exterior of the micelles, the results of these studies can be rationalized. In essence, then, this theory states that the greater the amount of water in the micelle interior the greater the aggregation tendency, and the greater the micelle exterior solvation the lesser the tendency for aggregation. The "intactness" assumption seems reasonable in view of the similarity of the water activity data for hexadecane, dodecane, and octane beyond the hexadecane turbidity point. If the hexadecane turbidity point corresponded to some kind of micelle destruction and consequent rearrangement of hexadecane, water, and AOT molecules, this similarity would be fortuitous.

The tendency for micellar aggregation should be inversely related to how well the outside of the micelles are solvated. The stronger and thicker the solvated layer, the farther apart the polar cores of the micelles will be kept. Therefore, the type of attractive forces (10) responsible for suspension and emulsion aggregation would be minimized.

Solvation would generally be expected to be exothermic. Therefore increase in temperature should generally result in a decrease in solvation. This is consistent with the temperature dependence observed for octane and dodecane (see Fig. 2). If there is no solvation one would expect that an increase in temperature would lessen the tendency for aggregation. This is compatible with the small but positive temperature dependence observed with the hexadecane system.[5]

From a purely statistical point of view, it is reasonable (11) to except that the lower hydrocarbons might better solvate the micelle than the higher hydrocarbons. The local thermodynamic activity of the externally exposed functional groups of the micelles is greater for higher hydrocarbon systems because the kinetic units of the solvent are larger for the higher hydrocarbons. Therefore, their tendency to mix with the solvent should be less; the externally exposed groups of the micelle have a greater tendency for intramicellar association in higher hydrocarbons.

The lack of distinct AOT concentration dependence (see Fig. 1) at first thought appears to be contradictory to this theory, but it must be kept in mind that these are concentrated systems and intermicellar interactions are likely to be important. Actually the hexadecane system, for which some low AOT concentration runs were made, does show (see Fig. 1) a rapid increase in the mole-mole ratio with decreasing AOT concentration at low AOT concentrations. This is consistent with the aggregation theory and the mass action concept.

Because for the lower hydrocarbons the activity of micellized water was essentially unity at the turbidity point, there was the likelihood of the presence of emulsion droplets of water in these cases. It is possible that these emulsion droplets were able to nucleate the aggregation in the lower hydrocarbons.[6] Further discussion of this possibility would be only speculation at this point, however.

Pharmaceutical Significance.—These thermodynamically stable islands of water in oil should provide a means of incorporating ionic and other highly polar drugs into essentially nonpolar vehicles. An example already cited above is the solubilization of sodium chloride in micellized water.

A useful application for these systems should be found in the development of ointments. It would seem that a relatively nondrying ointment base could be designed on these concepts. For highly polar drugs the ointment of this type would, furthermore, possess slow but prolonged release characteristics.

This technique of solubilization might be utilized in the preparation of sustained-action formulations in which the drug can be uniformly and reproducibly dispersed into a wax. Investigations of this possibility are now under way in our laboratories.

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[5] Temperature dependence for aggregation, *per se*, should be small since the energies involved are only the order of a few kT, where k = Boltzmann's constant and T = absolute temperature.

[6] Kinetics is not implied here, but that the droplets could provide surfaces for adsorption of micelles, and if some water is released by these micelles upon adsorption, this type of aggregation would perpetuate itself.

Physical Degradation of Emulsions Via the Molecular Diffusion Route and the Possible Prevention Thereof

By W. I. HIGUCHI and JAGDISH MISRA

The quantitative aspects of the rate of growth of the large particles and the rate of dissolution of the small particles in emulsions were theoretically examined for the case in which the process is diffusion controlled in the external phase. It was found that even if the miscibility of the dispersed phase is as low as about 1×10^{-7} Gm. per ml. the size distribution of micron size emulsion particles may change appreciably in 1 year. Furthermore, according to this mechanism the emulsion stability is approximately proportional to the particle volume. It is proposed that such unstable emulsions may be stabilized with respect to this process by the addition of small amounts of a third component which must distribute preferentially in the dispersed phase. Sedimentation experiments carried out with carbon tetrachloride emulsions in water with and without hexadecane and Nujol as additives support the theory.

WHEN one speaks of the instability of emulsions he most frequently has in mind the processes of creaming or sedimentation followed by coalescence, or the last process alone. Little thought, even at the qualitative level, has been given to examining the possible importance of molecular diffusion as a pathway in the degradation of emulsions.

This report presents a quantitative theoretical study of the various factors governing the degradation rate of emulsions when the process is strictly molecular diffusion controlled, i.e., when the coalescence rate is negligible. In Figs. 1A and 1B we illustrate the two processes of coalescence and molecular diffusion degradation, respectively. Coalescence then is the formation of a droplet from two smaller droplets, the significant step occurring when the two droplets get close enough to allow a contact of the droplet phases. Now in a molecular diffusion process two droplets may form a single droplet if conditions are such that one droplet is allowed to grow while the other dissolves. Hence, in the molecular diffusion process, the droplet phases are not required to come into contact.

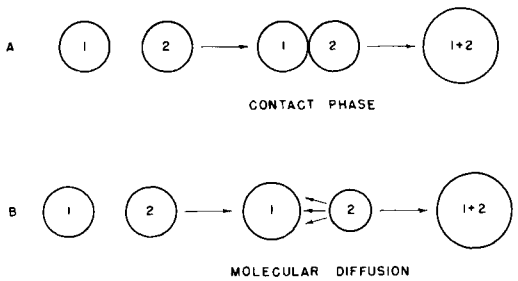


Fig. 1.—Illustration of emulsion degradation via coalescence (A) and via molecular diffusion degradation (B).

We can qualitatively visualize situations in which one mechanism will be more important than the other. For example an emulsion "stabilized" with respect to droplet contact by means of either a charge inducing agent or by means of a suitable protective colloid may yet yield to the mechanism of molecular diffusion degradation. On the other hand, an emulsion in which the internal phase is sufficiently immiscible in the external phase can only degrade by the droplet contact route.

THEORY

Case I. Simple Case of a Mixture of Two Sizes.—The differential equations for the rate of change of an arbitrary distribution of emulsion particle sizes cannot be solved by usual methods. For this reason it would be profitable to examine a specific simple case first so that a complete understanding of the situation in at least this one case might be obtained. We could then examine a more realistic case.

Consider an emulsion in which one finds particles of two sizes only. Let a_A and a_B be the radii of these particles and n_A and n_B their respective number concentrations. At time $t = 0$, let $a_A = a_{A0}$ and $A_B = a_{B0}$, with $a_{A0} < a_{B0}$.

The question we now would like to answer is, how do a_A and a_B vary with time? First let us examine the driving force responsible for degradation with which we are concerned.

It is well known (1) that the chemical potential of a component in a phase is a function of the molal interfacial area of the phase. Thus small crystals are more soluble than large ones, and the vapor pressures of small droplets are greater than the vapor pressures of large ones. Analogously, the miscibility of the internal phase material is a function of its molal interfacial area.

For a one-component droplet of radius r in equilibrium with the external phase solution the miscibility of the internal phase material with the external phase may be expressed by the following

Received April 28, 1961, from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication July 18, 1961.
Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

equation in the Henry's law region

$$c_r = c_\infty \exp(K/r) \qquad \text{(Eq. 1)}$$

Here c_r is the miscibility of the r-size droplet, c_∞ is the miscibility of the infinitely large droplet, and K is given by

$$K = (2\gamma M)/(\rho RT) \qquad \text{(Eq. 2)}$$

where γ is the interfacial tension of the interface between the two phases, M and ρ are the molecular weight and density of the internal phase material, R is the molar gas constant, and T is the temperature in °K.

It is clear from Eq. 1 that if more than one droplet size is present in an emulsion the chemical potential of the internal phase material in all of the droplets cannot be equal, i.e., the smaller particles will be thermodynamically unstable with respect to the large ones. Thus the smaller ones will tend to dissolve, and the larger ones will tend to grow at the expense of the former.

Let us now examine the mechanism of the rate process which we call molecular diffusion degradation. For a quantitative analysis of the problem we must assume that the system is dilute, i.e., a few per cent or less volume fraction of the internal phase.[1] Also we must assume that the miscibility of the internal phase material in the external phase is low, i.e., $\lesssim 1\%$ or so. Another simplifying assumption is that each particle must seek an environment which is both a time average and a space average distribution of other particles. This is a good assumption for not too viscous emulsions (dilute oil-in-water or dilute water-in-light oil emulsions) but not so good for, say, most heavy base ointments. Even for the latter however, to the extent that we are interested in approximate degradation rates, the assumption does not endanger our conclusions.

Now the rate of dissolution or the rate of growth of a sphere of radius a may be expressed (2) by the following equation when the process is diffusion controlled in the external phase and when $(c_s - c_o)$ is smaller than *ca.* 1×10^{-2} Gm. per ml.

$$G = 4\pi Da(c_s - c_o) \qquad \text{(Eq. 3)}$$

Here G is the rate, D is the molecular diffusion coefficient of the internal phase material in the external phase, c_s is the miscibility of the dissolving or growing phase, and c_o is the concentration of the material at some distance from the sphere, large compared to a.

Implicit in the derivation of Eq. 3 are several assumptions which now must be discussed. First, Eq. 3 assumes that no interfacial barrier is present. Studies (3) have indicated that for micron-size droplets or greater, mass transfer between two liquid phases is governed primarily by diffusion rates in the external phase. Even relatively strong interfacial monolayer films provided by conventional low molecular weight surfactants probably do not affect these rates (3). Thick viscous films provided by polymeric surfactants or by polymolecular films of low molecular weight surfactants may be important, however. There is a great need for good experimental work to solve these problems satisfactorily. We can only state at the present that in many, and in probably most, cases interfacial barriers against molecular mass transfer are not important in emulsion systems. Second, Eq. 3 assumes that hydrodynamic effects are not important. Nielsen's recent studies (2) show that fluid flow effects upon dissolution rates or growth rates caused by sedimentation or creaming of aqueous emulsion (density difference between particle and water less than unity) are unimportant for particle sizes *ca.* 25 μ or less. The same conclusions apply to moderately agitated systems where local fluid accelerations and decelerations do not exceed by very much that due to gravity. By the same type of analysis it can be shown that velocity gradients in the system up to about 10 sec.$^{-1}$ remain unimportant.

Now c_s in Eq. 3 is given by Eq. 1, so that for particles of size a_A

$$c_s = c_A = c_\infty \exp\left(\frac{K}{a_A}\right) \qquad \text{(Eq. 4)}$$

and for particles of size a_B

$$c_s = c_B = c_\infty \exp\left(\frac{K}{a_B}\right) \qquad \text{(Eq. 5)}$$

We get by combining Eqs. 3, 4, and 5

$$G_A = 4\pi Da_A\left[c_\infty \exp\left(\frac{K}{a_A}\right) - c_o\right] \qquad \text{(Eq. 6)}$$

and

$$G_B = 4\pi Da_B\left[c_\infty \exp\left(\frac{K}{a_B}\right) - c_o\right] \qquad \text{(Eq. 7)}$$

which are the rates of dissolution (positive G) or growth (negative G) for particles of sizes a_A and a_B, respectively. It is a result of the assumption that all particles seek an average environment which makes Eqs. 6 and 7 applicable. Otherwise there should be a distribution of dissolution or growth rates even for particles of the same initial size.

We may now obtain a further relationship for c_o on the basis of the mass conservation law. Clearly mass balance requires that

$$n_A G_A + n_B G_B + (d\bar{c})/dt = 0 \qquad \text{(Eq. 8)}$$

where t is the time and $\bar{c}$ is the average amount of dissolved internal phase material in the external phase per unit volume of emulsion. Combining Eqs. 6, 7, and 8 we get

$$4\pi Da_A n_A\left[c_\infty \exp\left(\frac{K}{a_A}\right) - c_o\right] + 4\pi Da_B n_B\left[c_\infty \exp\left(\frac{K}{a_B}\right) - c_o\right] + d\bar{c}/dt = 0 \qquad \text{(Eq. 9)}$$

It can be easily shown from our final results that $d\bar{c}/dt$ is negligible in comparison to the other two terms. This greatly simplifies our task, since then from Eq. 9 we may write

$$c_o = \frac{c_\infty[n_A a_A \exp(K/a_A) + n_B a_B \exp(K/a_B)]}{n_A a_A + n_B a_B} \qquad \text{(Eq. 10)}$$

[1] However even up to high concentrations (~40%), the rates will probably not increase by more than a factor of two or so.

Now if the exponential terms in Eq. 10 are expanded in series form according to

$$\exp(x) = 1 + x + (x^2/2) + x^3/6 + \ldots \quad \text{(Eq. 11)}$$

we get from Eq. 10

$$c_o = c_\infty \left[1 + \frac{K(n_A + n_B)}{n_A a_A + n_B a_B} + \text{higher order terms}\right] \quad \text{(Eq. 12)}$$

When $K/a << 1$, as it is for emulsions in the size range of interest to us, the higher order terms are always negligible.

Now the c_o expression may be substituted into Eqs. 6 and 7. The exponentials in the resulting equations may be expanded again according to Eq. 11 to give

$$G_A = \frac{4\pi D c_\infty K n_B (a_B - a_A)}{n_A a_A + n_B a_B} \quad \text{(Eq. 13)}$$

and

$$G_B = \frac{-4\pi D c_\infty K n_A (a_B - a_A)}{n_A a_A + n_B a_B} \quad \text{(Eq. 14)}$$

Because the rate of growth or dissolution of a particle must be equal to its rate of change of mass, we may also write

$$G_A = -4\pi\rho a_A^2 \frac{da_A}{dt} \quad \text{(Eq. 15)}$$

and

$$G_B = -4\pi\rho a_B^2 \frac{da_B}{dt} \quad \text{(Eq. 16)}$$

When these last two equations are combined with Eqs. 13 and 14 we get

$$\frac{da_A}{dt} = \frac{D c_\infty K}{\rho a_A^2}\left[\frac{n_B (a_B - a_A)}{n_A a_A + n_B a_B}\right] \quad \text{(Eq. 17)}$$

and

$$\frac{da_B}{dt} = \frac{D c_\infty K}{\rho a_B^2}\left[\frac{n_A (a_B - a_A)}{n_A a_A + n_B a_B}\right] \quad \text{(Eq. 18)}$$

These Eqs. 17 and 18 give the rates of change of the radii of the two sizes in the emulsion.

In Fig. 2 plots of a_A and a_B are presented for the case $n_A = n_B, a_{Ao} = 0.5\ \mu$, and $a_{Bo} = 1.0\ \mu$. Here

$$t' = D c_\infty K t/\rho \quad \text{(Eq. 19)}$$

The curves in Fig. 2 were obtained by numerically solving Eqs. 17 and 18. These results show that at $t' \sim 9 \times 10^{-14}$, all of the originally 0.5 μ particles have completely disappeared. By means of Eqs. 19 and 2 let us see how small c_∞ must be in this case in order to have a_A change only slightly, say 10%. We have then

$$c_\infty = \frac{\rho^2 t' RT}{2Dt\gamma M} \quad \text{(Eq. 20)}$$

Let us select $t' = 4 \times 10^{-14}$ (about 10% change in a_{Ao}), $t = 3 \times 10^7$ seconds (1 year's time), $M = 100$, ρ = unity, $D = 5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ (water viscosity in the external phase), and $\gamma = 1.0$ dyne cm.$^{-1}$. Insertion of these values into Eq. 20 gives $c_\infty = 3 \times 10^{-8}$ Gm./ml. This rather low upper limit value for c_∞ indicates that this mode of emulsion degradation can be a very important one. Thus substances in the micron size range with $c_\infty > 3 \times 10^{-8}$ Gm./ml. may undergo appreciable change in size distribution.

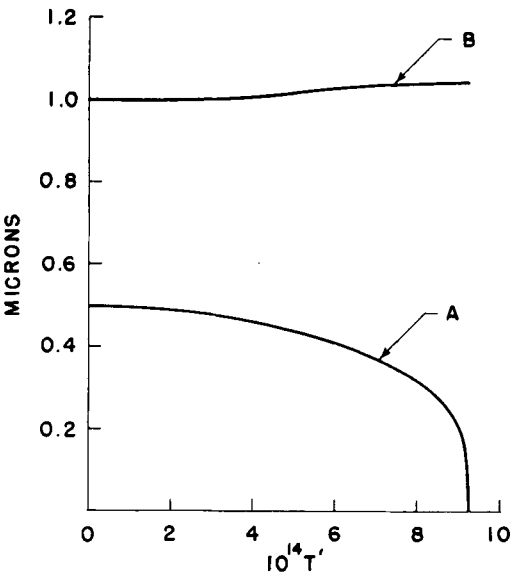


Fig. 2.—The degradation of an emulsion initially composed of a mixture of 0.5 μ and 1 μ radius droplets of equal number concentration. Curves A and B give droplet diameters as function of time (see Eq. 19 for definition of t').

Examination of Eq. 19 shows that the situation with respect to degradation can be improved by decreasing D which might be accomplished by increasing the viscosity of the external phase since, approximately

$$D = \frac{kT}{6\pi\eta\alpha} \quad \text{(Eq. 21)}$$

where η = viscosity of the external phase, α = molecular radius of diffusing material, and k = Boltzmann's constant.

Since the rate is proportional to γ, another way to retard degradation is to reduce γ by means of a suitable surfactant. However, one must avoid solubilization of the internal phase material in the external phase when using surfactants; otherwise the increase in c_∞ may offset the decrease in γ. Most water-oil interfacial tensions with surfactants are in the 0.1 to 10 dyne per cm. range.

The calculation for c_∞ just presented applies to the mixture of the 0.5 and 1.0 μ radii particles. If the emulsion is a mixture of 0.05 and 0.10 μ radii particles, the corresponding changes will occur a thousand times faster; i.e., the particle stability is proportional to the cube of its radius. Hence $c_\infty < 3 \times 10^{-11}$ Gm./ml. is the criterion for the 0.05–0.10 μ mixture, everything else remaining the same. This conclusion is apparent from Eqs. 17 and 18.

A careful examination of Eqs. 17 and 18 reveals that control of the size distribution in the preparation of the emulsion will not be too helpful with respect to the retarding of molecular diffusion degradation unless the distribution is made very nar-

row. Whether a_B is initially only a factor of two greater than a_A or whether it is many times greater makes only about a factor of two difference in the disappearance rate of the smaller particles. On the other hand, if $(a_B - a_A)$ is made very much smaller than a_A the rate will be inversely proportional to the narrowness of the distribution. Since most emulsions cannot be prepared with distributions so narrow, the narrowness of the distribution is generally not an important factor.

Fictitious as it may be, the emulsion composed of two particle sizes, $a_{Ao} = 0.5$ and $a_{Bo} = 1.0\ \mu$, of equal number concentration gives us a quantitative "feel" for the problem. The preceding analysis clearly shows that this route of degradation cannot be overlooked in emulsion stability studies.

Case II. More Complex Single Component Droplet Case.—To analyze a more realistic size distribution consider the emulsion with an initial ($t = 0$) distribution of radii following the law

$$n_i\, da_i = \frac{\bar{K}}{a_i^3}\, da_i \qquad \text{(Eq. 22)}$$

over the range $a_m \leq a_i \leq a_n$ at $t = 0$. For other, $t = 0$, a_i values assume $n_i = 0$. Here n_i is the concentration of particles in the size range between a_i and $a_i + da_i$ at $t = 0$, and $\bar{K}$ is a constant. The distribution given by Eq. 22 is a good approximation to the log-normal distribution.

The complexity of this problem limits the calculations to only the initial rate of degradation of the system. The rate of dissolution or growth of the ith particle at zero time is (analogous to Eqs. 6 and 7)

$$G_i = 4\pi\, Da_i \left[c_\infty \exp\left(\frac{K}{a_i}\right) - c_o \right] \qquad \text{(Eq. 23)}$$

where c_o is defined by a relationship similar to Eq. 10 but more general, *viz.*

$$\int_{am}^{an} 4\pi n_i Da_i \left[c_\infty \exp\left(\frac{K}{a_i}\right) - c_o \right] da_i = 0 \qquad \text{(Eq. 24)}$$

As before, the $d\bar{c}/dt$ term is neglected in deriving Eq. 24.

Substituting Eq. 22 into Eq. 24 and integrating for $t = 0$, we get

$$c_o = c_\infty \left[1 + \frac{K}{2}\left(\frac{1}{a_n} + \frac{1}{a_m}\right) \right] \qquad \text{(Eq. 25)}$$

with the neglect of higher order terms. Now if Eqs. 25 and 23 are combined we obtain

$$G_i = 4\pi\, Da_i\, c_\infty\, K\left(\frac{1}{a_i} - \frac{1}{2a_n} - \frac{1}{2a_m}\right) \qquad \text{(Eq. 26)}$$

for the ith particle at zero time. Equating G_i to the rate of change of mass of the ith particle

$$G_i = -4\pi\rho {a_i}^2 \frac{da_i}{dt} \qquad \text{(Eq. 27)}$$

and setting $a_i = a_m$ we get

$$\frac{da_m}{dt} = -\frac{Dc_\infty K}{2\rho a_m}\left(\frac{1}{a_m} - \frac{1}{a_n}\right) \qquad \text{(Eq. 28)}$$

which is the expression for the initial rate of size decrease of the smallest particles in the distribution.

Setting $a_i = a_n$ we get

$$\frac{da_n}{dt} = -\frac{Dc_\infty K}{2\rho a_n}\left(\frac{1}{a_n} - \frac{1}{a_m}\right) \qquad \text{(Eq. 29)}$$

which is the expression for the initial rate of growth of the largest particles in the distribution. It must be remembered that Eqs. 28 and 29 apply to only the initial rates while Eqs. 17 and 18 are valid for all times.

The quantitative similarity between Eqs. 28 and 29 and Eqs. 17 and 18 shows that a system with a continuous distribution of particle sizes does not behave too differently from the simple system consisting of only two sizes as far as the degradation rate is concerned. Thus, the general quantitative conclusions deduced for Case I are approximately applicable to more complex cases.

Case III. The Two Component Droplet Case.—While for both case I and case II the internal phase was treated as one component, in general one should consider the miscibility of the external phase material with the internal phase. Let us briefly outline the problem and discuss the conclusions for the case in which the external phase material is only slightly (say less than about 1%) miscible with the internal phase. The general case is much more difficult to analyze.

Figure 3 illustrates the problem. Component x makes up the bulk of the internal phase and component y makes up most of the external phase. c_{xl} and c_{yl} are the concentrations of the two components in the particle, c_{xs} and c_{ys} are the concentrations at the interface but on the external phase side, and c_{xo} and c_{yo} are the concentrations at some large distance from the particle.

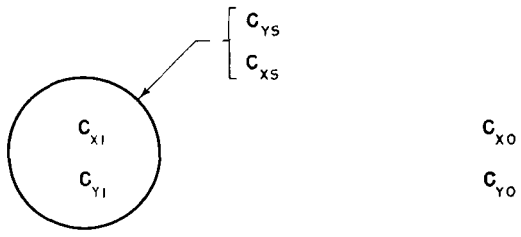


Fig. 3.—The two-component emulsion system. See text.

We may write for a particle of radius a

$$c_{xs} = k_x c_{xl} \exp\left(\frac{K_x}{a}\right) \qquad \text{(Eq. 30)}$$

and

$$c_{ys} = k_y c_{yl} \exp\left(\frac{K_y}{a}\right) \qquad \text{(Eq. 31)}$$

where k_x and k_y are the respective distribution coefficients, $K_x = 2\gamma \bar{V}_x/RT$, and $K_y = 2\gamma \bar{V}_y/RT$, with $\bar{V}_x$ and $\bar{V}_y$ the partial molal volumes of components x and y in the droplet. Equations 30 and 31 are simply the two component versions of Eq. 1. Both k_x and k_y can be expected to remain constant if each of the phases remain dilute with respect to one of the components (Raoult's law and Henry's law).

While we will not present the arguments, it can

be shown by Eqs. 30 and 31 and by the other conditions assumed above that the rate of growth or dissolution of the droplets in the emulsion is entirely controlled by the rate of diffusion of component x only. Furthermore it can be shown that

$$c_{ys} \simeq c_{yo} \qquad \text{(Eq. 32)}$$

in the steady state.

Therefore we may write

$$G_x = 4\pi D_x a\,(c_{xs} - c_{xo}) \qquad \text{(Eq. 33)}$$

for the rate of diffusion of component x toward or away from the particle. This becomes

$$G_x = 4\pi D_x a\left[k_x c_{xl} \exp\left(\frac{K_x}{a}\right) - c_{xo}\right] \qquad \text{(Eq. 34)}$$

when Eq. 30 is substituted for c_{xs}. Now because of Eq. 32

$$G_x = -4\pi a^2 c_{xl}\frac{da}{dt} \qquad \text{(Eq. 35)}$$

Then Eqs. 34 and 35 may be handled in the same way as was done in case I and the change in particle size may be evaluated as a function of time.

We may conclude that the two component droplet emulsion system behaves essentially the same as the one component droplet emulsion when the amount of the second component in the droplet is low, the second component being the predominant component of the external phase. This assumes, of course, that the appropriate γ and K_x are employed.

Case IV. Special Case of the Three Component Emulsion.—We shall not attempt a discussion of the general three component case. Let us, however, examine a special three component case which is of considerable importance to the question of emulsion stability.

In Fig. 4 the three component case is illustrated with the same symbolism as that used in Fig. 3 for the two component case but with addition of z values.

As before, let x and y represent the major internal phase and the major external phase components, respectively, with z being a minor component in both phases. As was found in case III (two component case) let the effect of y be negligible as far as the rates of change of the droplet sizes are concerned. These statements mean that

$$\left.\begin{aligned} &c_{yl} \ll c_{xl} \\ &c_{ys} \gg c_{xs} \\ &c_{yo} \gg c_{xo} \\ &c_{zs} \ll c_{ys} \\ &c_{zo} \ll c_{yo} \\ &c_{zl} \ll c_{xl} \end{aligned}\right\} \text{conditions (Eq. 36)}$$

and that

$$c_{ys} \simeq c_{yo} \text{ always}$$

We may write (as for the two component case)

$$c_{xs} = k_x c_{xl} \exp\left(\frac{K_x}{a}\right) \qquad \text{(Eq. 37a)}$$

and

$$c_{zs} = k_z c_{zl} \exp\left(\frac{K_z}{a}\right) \qquad \text{(Eq. 37b)}$$

Here k_x and K_x have the same meanings as before,

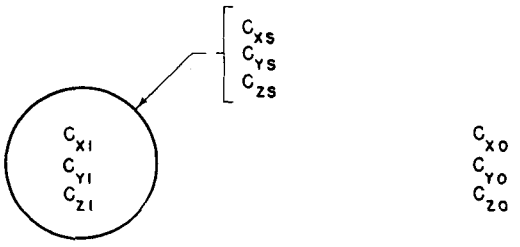


Fig. 4.—The three-component emulsion system. See text.

k_z is the corresponding distribution coefficient for the z component, and $K_z = 2\gamma \bar{V}_z/RT$ with $\bar{V}_z$ being the partial molal volume of component z in the droplet. The validity of Eqs. 37a and 37b may be easily demonstrated if it can be assumed that z does not interact with y to form a compound or complex in the internal phase and that z and x do not appreciably interact with each other in the external phase. In other words, for Eqs. 37a and 37b to apply, the solvent properties of each phase must be determined primarily by the major component in that phase. In the other extreme, i.e., when complex or compound formation between y and z in the internal phase or between x and z in the external phase occurs, more complex relationships than Eqs. 37a and 37b must be employed. However, even for these cases the general conclusions of this section will apply provided that the variations in k_x and k_y are taken into account.

Following the results for case III we may conclude that the condition, $c_{ys} \simeq c_{yo}$, is established very rapidly in any system for which $c_{ys} \neq c_{yo}$ initially. Then a period, τ, will ensue during which a pseudo-steady state is established. In the pseudo-steady state the rate of degradation of the system will depend upon either the rate of diffusion of x or that of z, or possibly upon both of these rates. If $k_x << k_z$ and if the diffusion coefficients for x and y are about the same, the degradation rate will not be markedly different from the one-component case. On the other hand, if $k_z << k_x$ the diffusion rate of z will govern the degradation rate of the particle at $t > \tau$. Physically what happens is that the relatively very slow rate of diffusion of z permits the x component to remain essentially equilibrated among all the droplets. Therefore, changes in the system occur only as fast as the change in the distribution of the slowest diffusing component. Thus, the degradation rate may be retarded by a factor

$$K_p = \frac{k_x}{k_z} \qquad \text{(Eq. 38)}$$

after a time τ. This result is significant to the extent that a system with a very large K_p value may be employed to stabilize emulsions which otherwise would degrade if the third (z) component were absent.

Having now established that in the pseudo-steady state ($t > \tau$) the degradation rate depends primarily upon K_p, let us now examine the period $t \lesssim \tau$, when K_p is infinitely large. For this purpose consider the three component emulsion containing only two sizes of particles. The situation is illustrated in Fig. 5. The symbolism is the same as in

Fig. 4 except the third subscripts A and B refer to the two particle sizes.

The initial boundary conditions ($t = 0$) for the two kinds of particles A and B or the number concentrations n_A and n_B are

$$a_A = a_{Ao} \qquad a_B = a_{Bo} \qquad \text{(Eq. 39)}$$

with

$$a_{Ao} < a_{Bo}$$

let also

$$c_{zlA} = c_{zlA_o} \text{ and } c_{xlB} = c_{xlB_o} \qquad c_{zlA} = c_{zlA_o} \text{ and } c_{zlB} = c_{zlB_o} \qquad \text{(Eq. 40)}$$

at $t = 0$.

Now immediately after the emulsion is prepared component y equilibrates rapidly to give $c_{ysA} \simeq c_{ysB} \simeq c_{yo}$. The main process with which we are now concerned is then that of the diffusion of x out of the A particles and into the B particles until the equilibrium condition

$$c_{xsA} = c_{xsB} \qquad \text{(Eq. 41)}$$

is reached. Since we have chosen $K_p \simeq \infty$, none of the z can leave or enter the droplets.

A practical and meaningful way to present the analysis is to determine the necessary initial concentration of z in the internal phase, $c_{zlo} = c_{zlA_o} = c_{zlB_o}$, which permits only a small change in a_A (say 10%) during the period, τ.

Substituting Eq. 37a into Eq. 41 we get

$$k_x\, c_{xlA_\infty} \exp\left(\frac{K_x}{a_{A_\infty}}\right) \simeq k_x\, c_{xlB_\infty} \exp\left(\frac{K_x}{a_{B_\infty}}\right) \qquad \text{(Eq. 42)}$$

where $c_{xlA_\infty} = c_{xlA}$, $c_{xlB_\infty} = c_{xlB}$, $a_{A_\infty} \neq a_A$, $a_{B_\infty} = a_B$, all at $t >> \tau$. Upon expansion of the exponential, Eq. 42 gives

$$\frac{c_{xlA_\infty}}{c_{xlB_\infty}} = 1 + K_x \frac{(a_{A_\infty} - a_{B_\infty})}{a_{A_\infty}\, a_{B_\infty}} + \ldots \qquad \text{(Eq. 43)}$$

we may also write

$$\bar{T} = \nu_{zl}\, c_{zl} + \nu_{xl}\, c_{xl} \qquad \text{(Eq. 44)}$$

Where $\bar{T}$ is a constant, and ν_{zl} and ν_{xl} are the partial specific volumes of z and x in the internal phase, respectively. Equation 44 is a good assumption for dilute solutions as specified under conditions (Eq. 36). It is valid for systems in which Raoult's law is obeyed for x and Henry's law is obeyed for z, i.e., for the case in which solute-solute interactions are unimportant.

Then for $t >> \tau$

$$\bar{T} = \nu_{zl}\, c_{zlA_\infty} + \nu_{xl}\, c_{xlA_\infty} \qquad \text{(Eq. 45)}$$

and

$$\bar{T} = \nu_{zl}\, c_{zlB_\infty} + \nu_{xl}\, c_{xlB_\infty}$$

where c_{zlB_∞} and c_{zlA_∞} are the c_{zlB} and c_{zlA} values at $t >> \tau$.

It is also apparent that

$$c_{zlA_\infty} = \frac{a_{A_o}^3}{a_{A_\infty}^3} c_{zlo} \qquad \text{(Eq. 46)}$$

and

$$c_{zlB_\infty} = \frac{a_{B_o}^3}{a_{B_\infty}^3} c_{zlo}$$

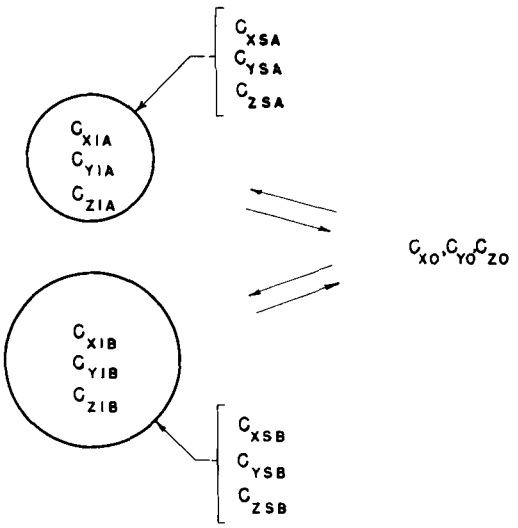


Fig. 5.—The three-component emulsion composed of a mixture of two sizes. See text for symbols.

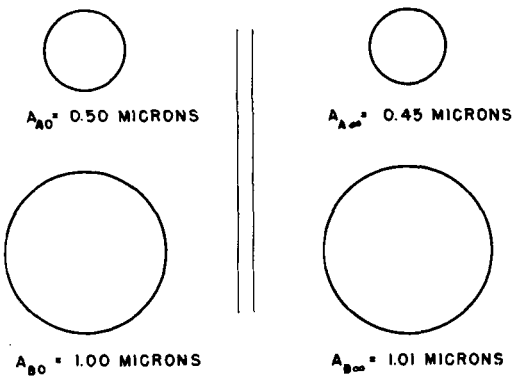


Fig. 6.—Illustration of the stabilization of an emulsion by a third component which is immiscible in the external phase. See text.

Now Eqs. 46, 45, and 43 may be combined to give

$$\frac{\bar{T} - \nu_{zl}(a_{A_o}^3/a_{A_\infty}^3)\, c_{zlo}}{\bar{T} - \nu_{zl}(a_{B_o}^3/a_{B_\infty}^3)\, c_{zlo}} = 1 + K_x \left(\frac{a_{A_\infty} - a_{B_\infty}}{a_{B_\infty}\, a_{A_\infty}}\right) \qquad \text{(Eq. 47)}$$

Then with the help of a final expression for the conservation of mass, *viz.*

$$n_A\, a_{A_o}^3 + n_B a_{B_o}^3 \simeq n_A a_{A_\infty}^3 + n_B a_{B_\infty}^3 \qquad \text{(Eq. 48)}$$

c_{zlo} may be evaluated.

Suppose as in case I we take $n_A = n_B$, $a_{Ao} = 0.5\ \mu$ and $a_{Bo} = 1.0\ \mu$ (See Fig. 6). Let us see what c_{zlo} must be so that $a_{A\infty} = 0.9\, a_{Ao}$, i.e., so that only a 10% change takes place during the period τ. Now $\bar{T} \sim$ unity, and for most organic compounds $\nu_{zl} \sim 1$. K_x will have values ranging from around 1×10^{-7} to 1×10^{-9}, depending primarily upon γ, since $\bar{V}_x \sim 100$, $RT \sim 2 \times 10^{10}$, and γ may generally vary from about 0.1 to 10 ergs per cm.^{2}. Employing these values with Eqs. 47 and 48 we find that $c_{zlo} \sim 3 \times 10^{-5}$ Gm. per ml. for $\gamma = 0.1$

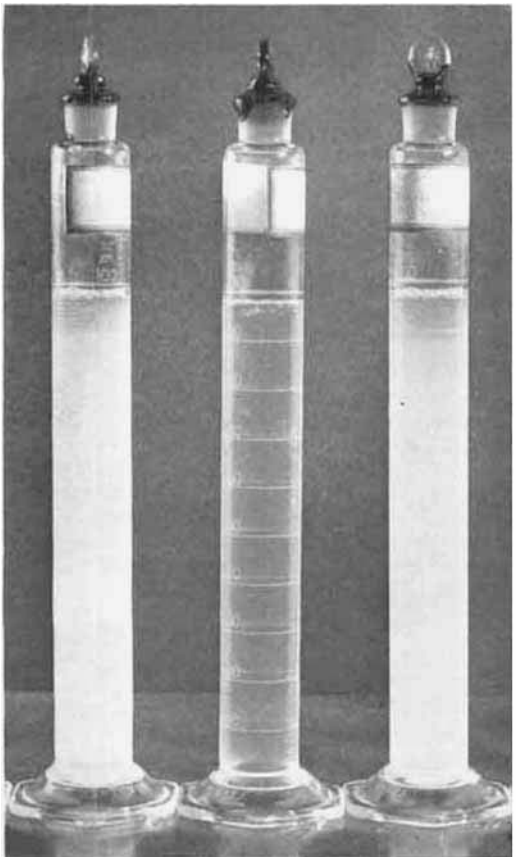


Fig. 7.—Photograph of 0.20% carbon tetrachloride-in-water emulsions containing 0.02% Aerosol OT, 12 hours after ultrasonic preparation. The center graduate, B, contains no other additive, while the graduates to the left, A, and right, C, contain 0.002% Nujol and hexadecane, respectively.

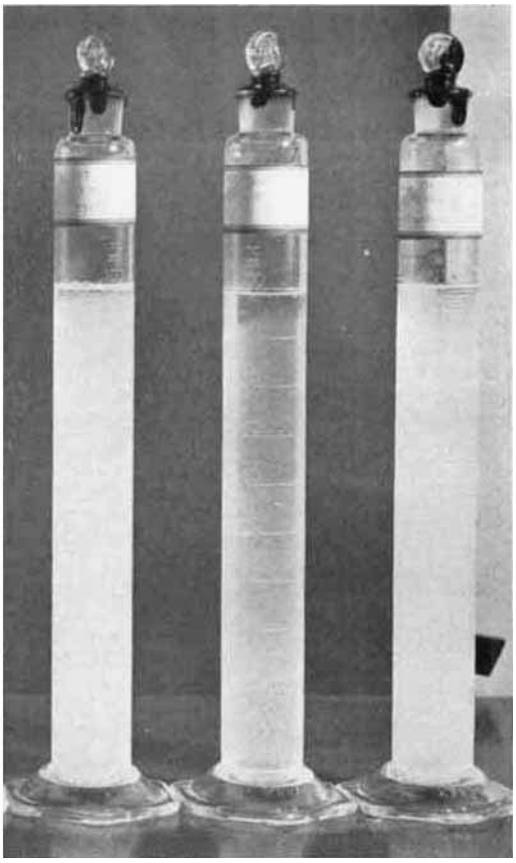


Fig. 8.—Photograph of 0.20% carbon tetrachloride emulsions containing 0.05% Aerosol OT 12 hours after preparation. Center graduate, B, contains no other additive while the graduates to the left, A, and right, C, contain 0.002% Nujol and hexadecane, respectively.

dyne per cm. and $c_{zlo} \sim 3 \times 10^{-3}$ Gm. per ml. for $\gamma = 10$ dynes per cm.

These values for c_{zlo} suggest that emulsions degrading via the molecular diffusion route may be stabilized by the incorporation of a small amount of a third component in the internal phase if this additive possesses a sufficiently large K_p value. About 1% additive should be more than ample for stabilizing most emulsions in the micron range. It is noteworthy that if γ is very low, say 0.01 dyne per cm., less than 0.01% additive is needed for stabilization.

For smaller size ranges Eq. 47 shows that c_{zlo} must be proportionately greater to provide the same stability. Therefore, if in the preceding calculations $a_{Ao} = 0.05\ \mu$ and $a_{Bo} = 0.10\ \mu$, the corresponding c_{zlo} values must be ten times greater.

EXPERIMENTAL STUDIES

Selection and Design of Experiment.—To test some of the ideas developed in the preceding sections of this report it was decided that dilute emulsions of carbon tetrachloride in water, stabilized against coalescence and aggregation, would be most appropriate for the following reasons: (*a*) According to Stokes' law the sedimentation rates of micron size particles of carbon tetrachloride in water would be great, i.e., *ca* 1×10^{-4} to 1×10^{-5} cm. sec.$^{-1}$. Thus, the presence or absence of submicron particles could be determined easily by visual observations. (*b*) The miscibility of carbon tetrachloride with water is high enough ($c_\infty \sim 1 \times 10^{-3}$ Gm. per ml.) so that submicron size particles in the presence of larger particles should disapear in about 1 hour according to our theory. (*c*) Additives such as hexadecane or Nujol may be employed to test the third component stabilization theory. These substances are miscible with carbon tetrachloride but possess very low miscibility with water, i.e., K_p is very great.

Materials and Procedure.—Carbon tetrachloride used was redistilled Baker reagent grade. Hexadecane was redistilled Eastman practical grade. Plough's pure Nujol was used without further purification. Aerosol OT (AOT) was purified (4) by fractional precipitation with methanol and a water-methanol solvent mixture. Water was redistilled with care to avoid any contamination by oil-miscible, water-immiscible materials.

The emulsions were prepared by ultrasonically emulsifying 0.2 ml. carbon tetrachloride (without

and with hexadecane or Nujol) in 100 ml. of water containing varying small amounts of Aerosol OT (0.02 to 0.10%). The carbon tetrachloride was pipetted into the detergent solutions in 100-ml. graduated cylinders (precleaned ultrasonically) with ground-glass stoppers. The stoppers were sealed with Cementyte to prevent any diffusion losses of the carbon tetrachloride vapor. The cylinders were then subjected to about 30 minutes of 40 kc. ultrasonic energy generated by the model DR-125AH Acoustica cleaning unit.[2] The emulsions were then removed from the ultrasonic bath and allowed to stand. The amount of sedimentation and the turbidity were observed from time to time for periods extending to several days.

Results.—In all cases where the additive, hexadecane or Nujol, was absent in the carbon tetrachloride the emulsion particles visibly coarsened relatively rapidly and settled. The rate of this process of coarsening and settling was greatest for the 0.02% AOT, in which case a clear liquid region at the top of several mm. formed in less than 1 hour. For the 0.10% AOT emulsions, the corresponding length of time was always several hours. Within about 8 hours nearly all of the particles in the 0.02% AOT emulsion settled completely, while it took several days in the case of the 0.10% AOT. Emulsions with intermediate concentrations (0.05%) of AOT showed intermediate rates.

In contrast to the above behavior, emulsions prepared from carbon tetrachloride containing 1% Nujol or hexadecane remained turbid longer and settled much more slowly than those emulsions free of these additives. It is noteworthy that the total hexadecane or Nujol in these cases was only 0.002%.

Figures 7 and 8 are photographs taken 12 hours after the preparation of emulsions for the 0.02 and 0.05% AOT, respectively. The center graduate, B, in each case is the emulsion without the additive; A and C containing Nujol and hexadecane, respectively. In the case of the 0.10% AOT, the situation was much like that of 0.05% AOT (Fig. 8) but after 4 days of standing.

The significant point here is that when the additive was present the small particles in the emulsion were most noticeably affected. Immediately after the preparation, the emulsions containing the same concentration of AOT had about the same appearance (turbidity). The large particles (1–10 μ) in the system, whether the additive was present or not, appeared to settle as expected (these can be seen in Figs. 7 and 8). The small particles (submicron) in the initial distribution were much more stable visibly, however, when the additive was present.

It was very difficult to ascribe these experimental findings to anything but molecular diffusion degradation and the prevention thereof. In all of these studies, visual examination of the sediment indicated that coalescence was unimportant, even for the 0.02% AOT cases. Even if some coalescence or aggregation were present, it is unreasonable to expect the presence of so little nonsurface-active ingredient as hexadecane or Nujol to influence coalescence or aggregation markedly over a wide range of surfactant concentration. Finally, the quantitative agreement (to an order of magnitude, of course) of the rate of degradation with theory should eliminate final doubts.

For emulsions not containing the additives, the increase in the apparent degradation rate with decreasing surfactant concentration may be explained on the following bases: first, the interfacial tension decreases with increasing surfactant concentration. The range of surfactant concentration employed, *viz.*, 0.02 to 0.10%, is commonly in the range in which γ changes rapidly. Thus, this is probably the main effect, following the theory. Second, it is possible that, at 0.10%, AOT may be involved in the formation of a polymolecular film at the interface. Cockbain (5) has proposed the multimolecular absorption idea to explain some of his findings on the aggregation of paraffin and benzene emulsions in AOT solutions. Mass transport through this film may be slow enough to affect the overall rate.

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Eserine Potentiation of Streptomycin Activity in Bacteria-Infected Embryonated Chicken Eggs

By VERNON A. GREEN†

Increases in per cent egg-day survival of eggs treated with combined streptomycin and eserine above the summation of the per cent egg-day survival of the two used separately were interpreted as potentiation of the antibacterial activity of the antibiotic. An attempt was made to correlate the cholinesterase activity of the bacteria with the eserine potentiation of the antibiotic activity of the streptomycin.

POTENTIATIONS of drug activity by eserine have been reported by Lewis (1), Greig (2, 3), and Green, *et al.* (4). Green and Davis (5) found that eserine potentiated the toxicity as well as the antibacterial activity of chloramphenicol in embryonated chick eggs.

In this study, possible eserine potentiation of the antibacterial activity of streptomycin, in embryonated chicken eggs infected with *Salmonella typhosa* WLR1207, *Proteus vulgaris* ATCC93, *Shigella dysenteriae* WLR194, or *Pseudomonus aeruginosa* ATCC9027, was investigated using the technique of McKenna and Taylor (6) for growing bacteria in yolk sacs. The criterion for determining potentiation was an increase in the per cent egg embryo (egg-day) survival above the summation of the per cent egg embryo survival of the antibiotic and eserine administered separately. Cholinesterase activity of the bacterial cells was determined by the method of Hall and Lucas (7); each unit of esterase activity being equal to 1 ml. of 0.01 *N* acetic acid formed in 10 minutes on the hydrolysis of acetylcholine solution at pH 8.

EXPERIMENTAL

Bacterial Inhibition.—Using the method reported by Green and Davis (5), embryonated chick eggs were infected deep in the yolk sac with 0.2 ml. of bacteria suspension to be used in each investigation. See Tables I–IV for concentration of microorganisms in seed suspension. After incubating at 37° for 6 hours, the eggs with living embryos designated for the investigation with each microorganism were divided into four groups of 40 eggs each. For each microorganism the groups were as follows: (*a*) control group, received only 0.2 ml./egg of sterile saline; (*b*) received streptomycin 0.1 mg./egg; (*c*) received streptomycin, 0.1 mg., and eserine salicylate, 0.0015 mg./egg; and (*d*) received eserine salicylate 0.0015 mg./egg.

The streptomycin, eserine, and eserine-streptomycin combinations were contained in 0.2 ml. of solution and were injected beneath the shell over the blood vessels of the embryo without rupturing the yolk sac. Upon sealing the shell with paraffin, the eggs were returned to the incubator and maintained at 37°.

After 7 hours the eggs were again candled and, thereafter, candled twice daily at 9:00 a.m. and 4:00 p.m. The embryo survivals were determined for 14 days, at which time any surviving embryos were destroyed, and the total day survivals for each group were recorded. The mean egg-day survival for each group was calculated; and using the mean egg-day survival of the saline controls used as the base or 100%, the mean egg-day survival of each group was reported as a percentage of the controls (see Tables I–IV).

Cholinesterase Determination.—Bacterial cells grown in beef heart infusion at 37° were packed by centrifuging, then washed and resuspended in sterile 0.9% saline three times. When approximately 3 ml. of packed cells were obtained, the cells were ground with sterile powdered glass in a bacteria mill and the bacterial protein extracted with 5% ammonium sulfate solution after the method of Nachmansohn (8). No attempt was made to concentrate the enzyme, but an amount of the extract equivalent to 1 ml. of the packed cells was used to determine the cholinesterase activity. The esterase activity per ml. of packed cells was as follows: (*a*) *Salmonella typhosa*, 0.021 units; (*b*) *Proteus vulgaris*, 0.050 units; (*c*) *Shigella dysenteriae*, 0.000 units; and (*d*) *Pseudomonus aeruginosa*, 0.025 units

RESULTS

In this study of eserine potentiation of the activity of streptomycin in *S. typhosa*-infected eggs, it was noted that the summation of the per cent egg-day survival of streptomycin- and eserine-treated eggs was approximately 90% less than the per cent survival of eggs receiving a combination of the two agents (Table I). Further, the difference in the per cent egg-day survival of *P. aeruginosa*-infected eggs treated with combined streptomycin and eserine and the summation of the per cent survival of the two used separately was approximately 120% (Table IV). The cholinesterase activity of the packed cells of the above two organisms corresponded closely in this study.

The increase in per cent egg-day survival in combined streptomycin- and eserine-treated eggs was greatest in eggs infected with *P. vulgaris*, approximately 500% (Table II). Eggs infected with *S. dysenteriae* treated with combined streptomycin and eserine showed a per cent egg-day survival approximately 110% below the summation of the two used separately (Table III). These findings were in

Received April 28, 1961, from the Pharmacology Division, The University of Texas, College of Pharmacy, Austin.
Accepted for publication July 6, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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TABLE I.—ESERINE POTENTIATION OF STREPTOMYCIN ACTIVITY IN CHICKEN EGGS INFECTED WITH *Salmonella typhosa*[a]

Agent Used	No. of Eggs	Egg-Day Survivals	% Egg-Day Survivals
Controls (saline)	40	56	100
Streptomycin, 0.1 mg.	40	176	300
Streptomycin, 0.1 mg., and eserine salicylate, 0.0015 mg.	40	288	500
Eserine salicylate, 0.0015 mg.	40	63	110

[a] Each egg infected with 0.2 ml. of a suspension of *Salmonella typhosa* containing 100,000 organisms per ml.

TABLE II.—ESERINE POTENTIATION OF STREPTOMYCIN ACTIVITY IN CHICKEN EGGS INFECTED WITH *Proteus vulgaris*[a]

Agent Used	No. of Eggs	Egg-Day Survivals	% Egg-Day Survivals
Controls (saline)	40	3	100
Streptomycin, 0.1 mg.	40	14	460
Streptomycin, 0.1 mg., and eserine salicylate, 0.0015 mg.	40	40	1300
Eserine salicylate, 0.0015 mg.	40	10	330

[a] Each egg infected with 0.2 ml. of a suspension of *Proteus vulgaris* containing 80,000 organisms per ml.

TABLE III.—ESERINE POTENTIATION OF STREPTOMYCIN ACTIVITY IN CHICKEN EGGS INFECTED WITH *Shigella dysenteriae*[a]

Agent Used	No. of Eggs	Egg-Day Survivals	% Egg-Day Survivals
Control (saline)	40	60	100
Streptomycin, 0.1 mg.	40	80	130
Streptomycin, 0.1 mg., and eserine salicylate, 0.0015 mg.	40	84	140
Eserine salicylate, 0.0015 mg.	40	72	120

[a] Each egg infected with 0.2 ml. of a suspension of *Shigella dysenteriae* containing 80,000 organisms per ml.

TABLE IV.—ESERINE POTENTIATION OF STREPTOMYCIN ACTIVITY IN CHICKEN EGGS INFECTED WITH *Pseudomonus aeruginosa*[a]

Agent Used	No. of Eggs	Egg-Day Survivals	% Egg-Day Survivals
Control (saline)	40	23	100
Streptomycin, 0.1 mg.	40	36	150
Streptomycin, 0.1 mg., and eserine salicylate, 0.0015 mg.	40	90	390

keeping with the cholinesterase activity of the packed cells, since *P. vulgaris* showed the highest esterase activity and *S. dysenteriae* showed the least esterase activity (no esterase activity by this method).

DISCUSSION

It appears from the data obtained in this study that eserine potentiation of streptomycin activity was greatest in eggs infected with *P. vulgaris* and least in eggs infected with *S. dysenteriae*. According to Schwartz, *et al.* (9), the minimal inhibitory concentration of streptomycin for *P. vulgaris* ATCC 93, *S. typhosa* WLRI 207, and *P. aeruginosa* ATCC 9027 is 37 mcg./ml., while the minimal inhibitory concentration of streptomycin for *S. dysenteriae* WLRI 94 is only 12.3 mcg./ml.

If the synergistic action of eserine on the antibacterial activity of streptomycin were linear, it appears that the increase in the per cent egg-day survivals of eggs infected with *P. vulgaris*, *S. typhosa*, and *P. aeruginosa* should have been of the same magnitude. Further, it appears that if the increase in embryo survivals in the above were due entirely to antibacterial action of the eserine salicylate, then the egg-day survival of the combined streptomycin- and eserine-treated eggs should have been at least equal to the summation of the two when used separately in *S. dysenteriae*-infected eggs.

Increases in the permeability of mouse brain and dog erythrocytes brought about by eserine was reported by Greig (2, 3). Further, this investigator found a 45% potentiation of the antibacterial activity of streptomycin on *E. coli* by eserine (4).

It appears probable that eserine, by some activity, may have increased the rate of absorption and, consequently, the concentration of the antibiotic in the tissues of the chick embryo. There appears, also, the probability that eserine, through its action with cholinesterase, may change the permeability of bacterial cells containing the enzyme, thus allowing the streptomycin to enter the cells more easily. At least, there appears to be some correlation between increases in per cent egg-day survivals and the cholinesterase content of the bacterial cells.

SUMMARY

In this study, the per cent egg-day survivals of eggs infected with *S. typhosa*, *P. vulgaris*, and *P. aeruginosa*, and treated with combined streptomycin and eserine were increased above the summation of the per cent egg-day survivals of streptomycin, 0.1 mg./egg, or 0.0015 mg./egg of eserine, used separately. The per cent egg-day survival of *S. dysenteriae*-infected eggs treated with combined streptomycin and eserine was less than the summation of the two agents used separately.

increase was greatest in *P. vulgaris*-infected eggs and least in *S. dysenteriae*-infected eggs. These findings appear to correlate with the esterase activity of the packed cells.

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Synthesis of Compounds Containing the Isoindoline Ring System

By ABOO SHOEB and JAMES E. GEARIEN

In an attempt to gain further knowledge concerning the pharmacological properties inherent in the isoindoline ring system, a number of compounds containing this ring system were synthesized and screened for biological activity. These compounds are analogs of biologically active compounds. Only the amide of N-[3-(2-isoindolinyl)propyl]-beta-alanine demonstrated appreciable biological activity. The carbon-hydrogen absorption bands present in the infrared spectra of these compounds were related to their chemical structure.

ALTHOUGH isoindoline is a relatively unstable compound, N-substituted isoindolines are quite stable, easily prepared, and provide convenient intermediates for synthesis. The ganglionic blocking action of 4,5,6,7-tetrachloro-2-(dimethylaminoethyl)isoindoline dimethochloride (1) and the hypertensive action of a series of the quaternary salts of hydrogenated bis-(2-isoindolinyl)alkanes (2) have been extensively studied. Few other compounds containing the isoindoline ring system have been reported to contain biological activity. It therefore appeared desirable to synthesize a number of analogs of biologically active compounds in which the isoindoline ring system is substituted for the amino or substituted amino group.

Three esters which contain the isoindoline ring system and which are analogs of procaine, Trasentine, and eucatropine were synthesized. These esters were 2-isoindolinylethyl 4-aminobenzoate hydrobromide, 2-isoindolinylethyl diphenylacetate hydrochloride, and 2-isoindolinylethyl *dl*-mandelate hydrochloride. The first of these esters was prepared by reacting 2-(2-hydroxyethyl)isoindoline with *p*-nitrobenzoyl chloride. The resulting 2-isoindolinylethyl *p*-nitrobenzoate was catalytically reduced to 2-isoindolinylethyl *p*-aminobenzoate. 2-Isoindolinylethyl diphenylacetate hydrochloride was similarly synthesized by the reaction of 2-(2-hydroxyethyl)isoindoline with diphenylacetyl chloride. 2-Isoindolinylethyl *dl*-mandelate was prepared by reacting silver mandelate with 2-(2-chloroethyl)-isoindoline.

In order to introduce the isoindoline ring system into a molecule having the prerequisites for antihistamine activity, 2-(2-isoindolinyl)ethyl benzhydryl ether was synthesized by reacting benzhydryl bromide with 2-(2-hydroxyethyl)-isoindoline in the presence of metallic sodium. It was isolated as its hydrochloride.

1,6-Bis-(2-isoindolinyl)hexane was prepared by the lithium aluminum hydride reduction of 1,6-hexane diphthalimide.

It also appeared desirable to introduce this ring system into compounds which might possess antitumor activity. 2-[2-Bis-(2-chloroethyl)-aminoethyl]isoindoline was synthesized by first reacting 2-(2-chloroethyl)isoindoline with diethanolamine to yield crude 2-[2-bis-(2-hydroxyethyl)aminoethyl]isoindoline which was then converted to the nitrogen mustard analog by treatment with thionyl chloride. The second possible antitumor agent, 1-[3-(2-isoindolinyl)-propyl]dihydrouracil, was prepared by the following series of reactions

Received April 28, 1961, from the Department of Chemistry, University of Illinois, College of Pharmacy, Chicago.
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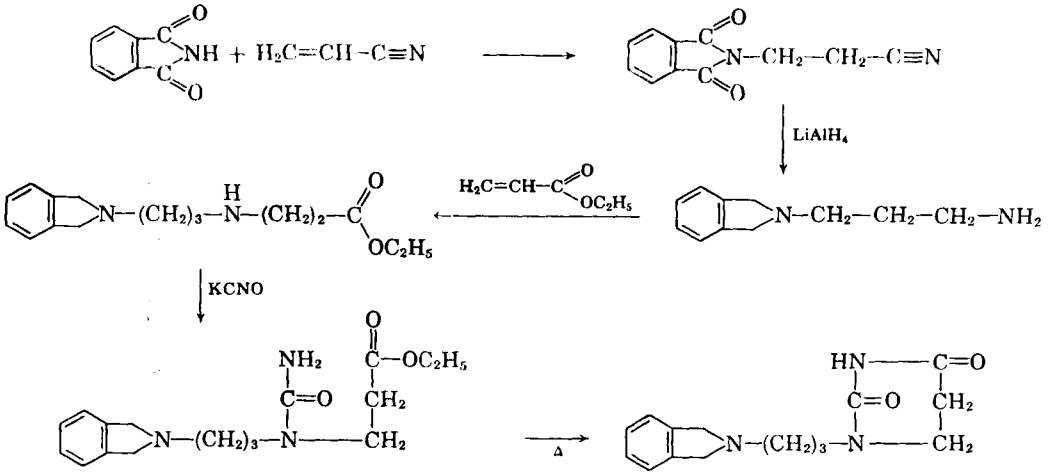
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It was found that 2-(3-aminopropyl)isoindoline could be added to acrylamide as well as to ethyl acrylate. The addition of 2-(3-aminopropyl)-isoindoline to acrylamide produced the amide of N-[3-(2-isoindolinyl)propyl]-beta-alanine. This compound is analogous to a number of compounds (3) which possess oxytocic activity.

N^1 - [4 - (2 - Phenylisoindolinyl)]sulfanilamide was prepared by the usual synthesis employed for N^1-substituted sulfanilamides.

The compounds prepared in this study were screened for biological activity. Only the amide of N - [3 - (2 - isoindolinyl)propyl] - beta - alanine showed appreciable activity. This compound stimulated the contractions of isolated uterine muscles.

Most of the compounds prepared were found to contain three carbon-hydrogen absorption bands in the 2700–3200 cm.$^{-1}$ region of their infrared spectra. Since absorption in this area of the spectrum is usually the result of the absorption by carbon-hydrogen bonds and since there was present in these molecules three distinct types of carbon-hydrogen bonds, it was felt that with the aid of several simple model compounds, analysis of this region of their spectra would be possible. From a study of the spectra of the compounds listed in Table I it appeared that the three bands were the result of absorption by the carbon-hydrogen bonds of the aromatic ring, the carbon-hydrogen bonds of the heterocyclic ring, and the carbon-hydrogen bonds of the alkyl chain attached to the nitrogen atom.

The bands appearing at 3025–3050 cm.$^{-1}$ result from absorption by aromatic carbon hydrogen bonds. When only a single aromatic ring is present in the compound, the band occurs at 3025 as a shoulder on the stronger peak at 2940 cm.$^{-1}$. However, the addition of a second phenyl group as in 2 phenylisoindoline, 2-phenyl-4-aminoisoindoline, or 1,6-bis(2-isoindolinyl)hexane, increases the intensity of this band and it occurs as a separate peak. The absorption bands at 2940 to 2975 cm.$^{-1}$ were definitely established. These appeared to be due to absorption of the carbon-hydrogen bonds of the alkyl groups connected to the heterocyclic nitrogen atom. Substitution of a phenyl group for the alkyl group, 2-phenylisoindoline and 2-phenyl-4-amino-isoindoline, resulted in the loss of absorption peaks in this area. Furthermore, the substitution of an ethyl group for the methyl group of 2-methylisoindoline gave rise to a doublet rather than a single peak.

It appeared likely that the absorption bands occurring in the portion of the infrared spectra 2720 and 2800 cm.$^{-1}$ might be caused by the absorption of carbon-hydrogen bonds of the methylene group of the heterocyclic ring. It was, therefore, desirable to obtain the spectra of an isoindoline ring system that did not contain carbon-hydrogen bonds in that ring. Since it was difficult to obtain a 1,1,3,3 tetra-substituted isoindoline, the spectra of the compounds previously synthesized were compared to the spectra of N-methylphthalimide. While the previously identified peaks at 3050 and 2975 cm.$^{-1}$ were pres-

TABLE I.—STUDY OF THE SPECTRA

Compound	C—H Bond Aromatic Ring	C—H Bond Hetero-cyclic Ring	C-Bond Alkyl Chain
2-Methylisoindoline	3025[a]	2775	2940
2-Ethylisoindoline	3025[a]	2800	2940–2975
2-Phenylisoindoline	3050	2750	...
2-Phenyl-3-amino-isoindoline	3040	2720	...
1,6-Bis-(2-isoindo-linyl)hexane	3050	2780	2940
N-Methylphthalim-ide	3050	...	2975

[a] Occurs as a shoulder on the peak at 2940 cm.$^{-1}$.

ent, the peak at 2720 to 2800 was missing. This indicates that the peak usually occurring in this area is due to absorption of the methylene group of the heterocyclic ring.

EXPERIMENTAL[1]

2-(2-Hydroxyethyl)isoindoline.—A solution of 9.0 Gm. (0.047 mole) of N-(2-hydroxyethyl)phthalimide (4) in 25 ml. of tetrahydrofuran was slowly added to a previously cooled slurry of 5.4 Gm. (0.047 mole) of lithium aluminum hydride in 100 ml. of anhydrous ether. After the addition was completed, the cold reaction mixture was decomposed with absolute alcohol. Water was then added until the aluminum oxide was completely precipitated. The resulting suspension was filtered and the clear filtrate dried over anhydrous sodium sulfate. After the removal of the ether, the residue was distilled. It boiled at 167–169°/15 mm. The distillate weighed 4.6 Gm. (60%).

von Braun and co-workers (5) prepared 2-(2-hydroxyethyl)isoindoline by the reaction of ethylene oxide on isoindoline. They reported the isolation of an oil boiling at 162–164°/12 mm.

2-Isoindolinylethyl *p*-Nitrobenzoate.—To a stirred solution of 6.5 Gm. (0.04 mole) of 2-(2-hydroxyethyl)isoindoline in 50 ml. of dry benzene was added, dropwise, a solution of 7.5 Gm. (0.04 mole) of *p*-nitrobenzoyl chloride in 25 ml. of dry benzene. The reaction mixture was refluxed for 2 hours and the solvent removed. The remaining oil was neutralized with 10% sodium hydroxide and then extracted with benzene. After evaporation of the solvent, 9.3 Gm. (75%) of yellow crystals was obtained. These were recrystallized from an alcohol-water mixture.

Anal.—Calcd. for $C_{17}H_{16}N_2O_4$: C, 65.37; H, 5.16; N, 8.97. Found: C, 65.18; H, 4.96; N, 8.78.

2-Isoindolinylethyl *p*-Aminobenzoate Hydrobromide.—The reduction of 2-isoindolinylethyl *p*-nitrobenzoate to 2-isoindolinylethyl 4-aminobenzoate was carried out by shaking a solution of 3.0 Gm. (0.009 mole) of the nitro compound in 75 ml. of ether with 0.3 Gm. of Raney nickel catalyst at 15 pounds of hydrogen pressure. When the theoretical quantity of hydrogen had been absorbed, the hydrogenation was discontinued and the catalyst removed by filtration. The filtrate was treated with anhydrous hydrogen bromide. The precipitated hydrobromide salt was filtered and recrystallized from absolute alcohol by precipitation with anhydrous ether. The pure compound weighed 1.2 Gm. (42%) and melted at 239–240°.

Anal.—Calcd. for $C_{17}H_{19}BrN_2O_2$: C, 56.21; H, 5.26; N, 7.70. Found: C, 56.00; H, 5.26; N, 7.80.

2-(2-Chloroethyl)isoindoline Hydrochloride.—To 8.6 Gm. (0.053 mole) of 2-(2-hydroxyethyl)isoindoline dissolved in 20 ml. of chloroform was added slowly a solution of 8 ml. of thionyl chloride in 15 ml. of chloroform. The reaction mixture, after being refluxed for approximately 15 minutes, crystallized. After cooling in ice, the reaction mixture was filtered and the resulting crystalline solid washed with chloroform and then with ether. After recrystallization from absolute alcohol by the addition of a few drops of anhydrous ether, a yield of 10.6 Gm. (91%) of a white crystalline solid was obtained. It melted at 191–192°.

Anal.—Calcd. for $C_{10}H_{13}Cl_2N$: C, 55.06; H, 6.00; Cl, 32.51. Found: C, 55.21; H, 5.87; Cl, 32.43.

This compound has been previously reported by Gump, *et al.* (6). Its preparation and physical constants were not reported.

2-Isoindolinylethyl *dl*-Mandelate Hydrochloride.—Silver mandelate was prepared by neutralizing 10.0 Gm. of *dl*-mandelic acid with dilute ammonium hydroxide and adding a 10% solution of silver nitrate until the precipitation of the silver salt was complete. The silver mandelate was removed by filtration, washed with water, alcohol, and finally ether.

A mixture of 4.2 Gm. (0.016 mole) of silver mandelate, 3.5 Gm. (0.020 mole) of 2-(2-chloroethyl)isoindoline hydrochloride, and 20 ml. of dry benzene was refluxed for 8 hours. During this time the solution was protected from light. The resulting silver chloride was removed by filtration. Ether was added to the filtrate to produce a gummy mass which solidified upon standing in the refrigerator overnight. The solvent was decanted and the residue dissolved in absolute alcohol. The hydrochloride precipitated upon the addition of anhydrous hydrogen chloride. After recrystallization from isopropyl alcohol, 3.0 Gm. (58%) of a white solid melting at 151–152° was obtained.

Anal.—Calcd. for $C_{18}H_{20}ClNO_3$: C, 64.75; H, 6.00; Cl, 10.64; N, 4.20. Found: C, 64.27; H, 6.09; Cl, 10.28; N, 4.37.

2-Isoindolinylethyl Diphenylacetate Hydrochloride.—To a stirred solution of 7.0 Gm. (0.043 mole) of 2-(2-hydroxyethyl)isoindoline in 25 ml. of dry benzene was added slowly a solution of 10.0 Gm. (0.043 mole) of diphenylacetyl chloride (7) in 15 ml. of dry benzene. The reaction mixture was refluxed on a steam bath for 2 hours. After removal of the solvent on a flash evaporator, the residue was recrystallized from isopropyl alcohol. The pure compound melted at 194–195° and weighed 15 Gm. (92%).

Anal.—Calcd. for $C_{24}H_{24}ClNO_2$: C, 73.18; H, 6.14; Cl, 9.02; N, 3.56. Found: C, 73.16; H, 6.21; Cl, 9.00; N, 3.70.

2-(2-Isoindolinyl)ethyl Benzhydryl Ether Hydrochloride.—2-(2-Hydroxyethyl)isoindoline (8.0 Gm., 0.049 mole) was dissolved in 20 ml. of dry benzene. To this solution was added 1.3 Gm. (0.055 mole) of sodium cut in very small pieces. After the mixture was refluxed for 1 hour, a solution of 13.7 Gm. (0.055 mole) of benzhydryl bromide (8) was added and the resulting solution refluxed for 4 hours. The reaction mixture was filtered to remove the precipitated sodium bromide. After evaporation of the solvent in a flash evaporator, the residue was distilled. It boiled at 195°/0.35 mm. The viscous reddish-brown distillate was dissolved in the least possible amount of benzene and the solution was allowed to stand overnight. A small amount of tetraphenylethane (m.p. 200°) crystallized and was removed by filtration. An equal volume of ether was added to the filtrate and the resulting solution was saturated with anhydrous hydrogen chloride.

[1] Melting points are uncorrected. Microanalyses are by Weiler and Strauss, Oxford, England.

The hydrochloride precipitated and was removed by filtration. After recrystallization from absolute ethanol, 0.7 Gm. (38%) of a white solid melting at 204–204.5° was obtained.

Anal.—Calcd. for $C_{23}H_{29}ClNO$: C, 75.51; H, 6.56; Cl, 9.71. Found: C, 75.66; H, 6.72; Cl, 9.86.

1,6-Hexamethylenediphthalimide.—To 10.0 Gm. (0.085 mole) of 1,6-hexamethylenediamine in a round bottomed flask was added 25.5 Gm. (0.17 mole) of phthalic anhydride. After the vigorous reaction had subsided, the mixture was heated for 45 minutes at 100°, after which the temperature was increased to 160° for 10 minutes. The solid residue was treated with boiling 50% alcohol and the insoluble precipitate was removed by filtration. After recrystallization from tetrahydrofuran the crystals melted at 178–179°. It weighed 30.0 Gm. (97%).

Anal.—Calcd. for $C_{22}H_{20}N_2O_4$: C, 70.21; H, 5.32; N, 7.44. Found: C, 70.13; H, 5.31; N, 7.65.

1,6-Bis-(2-isoindolinyl)hexane Dihydrochloride.—A solution of 6.6 Gm. (0.018 mole) in 20 ml. of tetrahydrofuran was added, dropwise, to a slurry of 4.0 Gm. of lithium aluminum hydride in 75 ml. of anhydrous ether. The reaction mixture was refluxed for 15 minutes and then the reagent was decomposed by the addition of ethanol. Water was carefully added to precipitate the alumina which was filtered. The clear filtrate was evaporated to dryness and the resulting solid dissolved in benzene and treated with an equivalent of an alcoholic solution of hydrogen chloride. The precipitated hydrochloride, after recrystallization from methanol, melted at 297–298°. The crystals weighed 3.5 Gm. (50%).

Anal.—Calcd. for $C_{22}H_{30}Cl_2N_2$: C, 67.17; H, 7.63; N, 7.12. Found: C, 66.85; H, 7.13; N, 6.81.

2-[2-Bis(2-chloroethyl)aminoethyl]isoindoline Dihydrochloride.—To a 100-ml. mixture of equal parts alcohol and benzene was added 7.5 Gm. (0.034 moles) of 2-(2-chloroethyl)isoindoline hydrochloride and 4.6 Gm. (0.034 mole) of diethanolamine. The resulting solution was refluxed for 4 hours, after which the solvents were removed on a flash evaporator. The residue dissolved in 10 ml. of chloroform was added, dropwise, to a solution of 15 ml. of thionyl chloride in 15 ml. of chloroform. When the addition was completed the mixture was heated under reflux for 20 minutes and then cooled in an ice bath until crystallization was complete. The solid reaction product after filtration and recrystallization from a mixture of equal parts acetone and alcohol weighed 11.0 Gm. (90%) and melted at 270° (decompn.).

Anal.—Calcd. for $C_{14}H_{22}Cl_4N_2$: C, 46.66; H, 6.12; Cl, 39.45; N, 7.78. Found: C, 46.52; H, 6.26; Cl, 39.51; N, 7.85.

2-(3-Aminopropyl)isoindoline.—2-Cyanoethylphthalimide (9) (15.0 Gm. 0.087 mole) was placed in a Soxhlet thimble and extracted into a slurry of 10.6 Gm. of lithium aluminum hydride in 200 ml. of anhydrous ether. After the reaction was completed, the reaction mixture was cooled to 5°, decomposed first with absolute alcohol, and finally with sufficient water to precipitate the aluminum oxide. The suspension was filtered and the clear filtrate was dried over anhydrous sodium sulfate. The mixture was filtered and the solvent removed by distillation. The resulting oil was distilled under vacuum. The fraction distilling at 94–96°/0.6 mm. was collected. It weighed 9.8 Gm. (60%).

Anal.—Calcd. for $C_{11}H_{16}N_2$: C, 74.95; H, 9.15; N, 15.90. Found: C, 74.82; H, 9.34; N, 15.92.

The pure oil rapidly absorbed carbon dioxide from the air to form a solid carbonate. The free base was regenerated upon the addition of strong alkali.

N-[3-(2-Isoindolinyl)propyl]-beta-alanine Amide Dihydrochloride.—A solution of 1.8 Gm. (0.01 mole) acrylamide in 20 ml. of absolute ethanol was slowly added to a stirred solution of 2.1 Gm. (0.02 mole) of 2-(3-aminopropyl)isoindoline in 10 ml. of absolute ethanol. The mixture was allowed to stand for 1 week. After removal of the solvent in a flash evaporator, the dark residue was washed with anhydrous ether and then dissolved in absolute alcohol. The hydrochloride was precipitated by the addition of anhydrous hydrogen chloride. The resulting precipitate was recrystallized from a mixture of equal parts of isopropanol and 95% alcohol. The recrystallization gave 2.7 Gm. (85%) of a white crystalline precipitate that melted at 173–174°.

Anal.—Calcd. for $C_{14}H_{23}Cl_2N_3O$: C, 52.50; H, 7.24; Cl, 22.14; N, 13.13. Found: C, 52.43; H, 6.97; Cl, 22.31; N, 12.98.

N-[3-(2-Isoindolinyl)propyl]-beta-alanine Ethyl Ester.—A solution of 5.7 Gm. (0.057 mole) of ethyl acrylate in 10 ml. of absolute alcohol was added dropwise, with stirring, to a cooled solution of 10.0 Gm. (0.057 mole) of 2-(3-aminopropyl)isoindoline in 20 ml. of absolute alcohol. The mixture was allowed to stand at room temperature for 4 days, at which time the solvent was removed on a steam bath under *vacuum*. The residue distilled and a fraction weighing 14.2 Gm. (90%) and distilling at 156–158°/0.6 mm. was collected.

Anal.—Calcd. for $C_{16}H_{24}N_2O_2$: C, 69.53; H, 8.75; N, 10.15. Found: C, 69.28; H, 8.52; N, 10.11.

1-[3-(2-Isoindolinyl)propyl]dihydrouracil.—To a stirred solution of 8.8 Gm. (0.032 mole) of N-[3-(2-isoindolinyl)propyl]-beta-alanine ethyl ester in a mixture of 3 ml. of hydrochloric acid and 10 ml. of water was added, dropwise, a solution of 3.4 Gm. (0.042 mole) of potassium cyanate in 10 ml. of water. The resulting solution was stirred for 1 hour and then allowed to stand overnight at room temperature. During this time a second layer formed. The upper layer was separated and treated with sufficient alcohol to precipitate the potassium cyanate present completely. After filtration of the solid, the filtrate was evaporated to dryness. A solid remained which, after recrystallization from absolute alcohol, gave 2.2 Gm. (25%) of a white crystalline solid melting at 184–185°.

Anal.—Calcd. for $C_{15}H_{19}N_3O_2$: C, 65.91; H, 7.00; N, 15.38. Found: C, 65.63; H, 7.29; N, 15.38.

N-Phenyl-3-nitrophthalimide.—To 3-nitrophthalic anhydride (10) (66.0 Gm., 0.45 mole) was added, dropwise, 42.0 Gm. (0.45 mole) of redistilled aniline. After the addition was completed and the reaction had subsided, the reaction mixture was heated at 210° for 20 minutes. The resulting orange liquid solidified on cooling and was recrystallized from a mixture of equal parts of acetone and ethanol. It

yielded a yellow crystalline solid which melted at 136–138°. The yield was 107.0 Gm. (82%).

Anal.—Calcd. for $C_{14}H_8N_2O_4$: C, 62.68; H, 3.01; N, 10.52. Found: C, 62.43; H, 2.89; N, 10.32.

N-Phenyl-3-aminophthalimide.—N-Phenyl-3-nitrophthalimide (10.0 Gm., 0.037 mole) dissolved in 100 ml. of equal parts tetrahydrofuran and ethanol, was reduced over Raney nickel catalyst in a Parr hydrogenator at 30 pounds of hydrogen pressure. After approximately 8 hours of shaking, the theoretical quantity of hydrogen had been absorbed. The solvent was then removed on a flash evaporator and the solid residue was recrystallized from benzene to yield 7.5 Gm. (85%) of a white crystalline solid. It melted at 185–187°.

Anal.—Calcd. for $C_{14}H_{10}N_2O_2$: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.63; H, 4.02; N, 11.88.

2-Phenyl-4-aminoisoindoline.—A solution of 13.0 Gm. (0.054 mole) of N-phenyl-3-aminophthalimide in 100 ml. of tetrahydrofuran was added, dropwise, to a suspension of 6.2 Gm. of lithium aluminum hydride in 75 ml. of tetrahydrofuran. The addition required 45 minutes. The reaction mixture was then refluxed for an additional 10 minutes, cooled in an ice bath, and the excess lithium aluminum hydride decomposed with absolute alcohol. The alumina was precipitated by the careful addition of water and the reaction mixture was filtered. The filtrate was dried over anhydrous sodium sulfate. After removal of the solvent, a greenish solid remained. After recrystallization from ethanol it weighed 10.0 Gm. (87%) and melted at 123–124°.

Anal.—Calcd. for $C_{14}H_{14}N_2$: C, 79.96; H, 6.71; N, 13.33. Found: C, 79.72; H, 6.83; N, 13.55.

N^1-[4-(2-Phenylisoindolinyl)]-N^4-acetylsulfanilamide.—To a refluxing solution of 2.1 Gm. (0.010 mole) of 2-phenyl-4-aminoisoindoline and 0.8 Gm. (0.010 mole) of pyridine in 30 ml. of benzene was added, dropwise, a solution of 2.6 Gm. (0.011 mole) of 4-acetamidobenzenesulfonyl chloride in 20 ml. of equal parts benzene and acetone. After the addition was completed, the resulting solution was refluxed for 10 minutes. Upon cooling, an oil settled to the bottom of the flask. The supernatant liquid was decanted and the oil was washed with ether and finally with cold water. Upon standing it solidified and was recrystallized from 50% isopropanol to give 3.3 Gm. (81%) of a white crystalline solid. The solid melted at 230–231°.

Anal.—Calcd. for $C_{22}H_{21}N_3O_3S$: C, 64.84; H, 5.19; N, 10.31; S, 7.86. Found: C, 64.78; H, 5.55; N, 10.41; S, 7.51.

N^1-[4-(2-Phenylisoindolinyl)]sulfanilamide.—N^1-[4-(2-Phenylisoindolinyl)]-N^4-acetylsulfanilamide (2.8 Gm., 0.007 mole) was refluxed for 1 hour with a solution of 2 ml. of hydrochloric acid in 4 ml. of water. The reaction mixture was then neutralized with sodium carbonate and the precipitated product collected on a Buchner funnel. After recrystallization from isopropanol to which a few drops of acetone was added, 1.8 Gm. (69%) of a white crystalline solid was obtained. It melted at 234–236°.

Anal.—Calcd. for $C_{20}H_{19}N_3O_2S$: C, 65.73; H, 5.25; N, 11.49; S, 8.77. Found: C, 65.50; H, 5.51; N, 11.22; S, 8.68.

2-Methylisoindoline.—2-Methylisoindoline was prepared by the lithium aluminum hydride reduction of N-methylphthalimide, following the procedure employed for the preparation of 2-(2-hydroxyethyl)isoindoline. It distilled at 85–86°/15 mm. [lit. (11) b.p. 81–82°/13 mm.].

2-Ethylisoindoline.—The compound was prepared by the lithium aluminum hydride reduction of N-acetylphthalimide, following the procedure of Rabjohn, Drumm, and Elliott (12). A fraction distilling at 217–220° was collected [lit. (12) b.p. 219–220°].

2-Phenylisoindoline.—N-Phenylphthalimide was reduced with lithium aluminum hydride as described by Wittig, Closs, and Mindermann. It melted at 168–169° [lit. (13) b.p. 169–170°].

SUMMARY

A series of compounds containing the isoindoline ring system, which are analogs of biologically active compounds, were prepared and screened for pharmacological activity. Only the amide of N-[3-(2-isoindolinyl)propyl]-beta-alanine showed appreciable biological activity. This compound increased the contractions of the isolated guinea pig uterus.

The carbon-hydrogen absorption bands occurring in 2700–3200 $cm.^{-1}$ region of their infrared spectra were examined and related to their molecular structure. The absorption bands occurring from 3025 to 3050 were the result of absorption of carbon-hydrogen bonds in the aromatic ring. Those occurring in the 2940–2975 region were the results of absorption of carbon-hydrogen bonds in the alkyl chain attached to the heterocyclic nitrogen atom. The band occurring at 2750 to 2780 was caused by absorption of the methylene group in the heterocyclic ring.

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Polarographic Method of Analysis for Pharmaceuticals

By A. FRANCIS SUMMA

Polarographic methods for the determination of acetazolamide, chlorothiazide, and nitrofurantoin, and their application to pharmaceutical dosage forms, are described. These methods are satisfactory for the assay of pharmaceutical preparations official in U.S.P. XVI. The polarographic methods have the desired accuracy and precision for routine control procedures.

SINCE the introduction of polarography by Heyrovsky in 1920, there have been a number of reports in the literature concerning its application to pharmaceutical dosage forms (1–5). As with other new analytical techniques, there has been a tendency among some of the more enthusiastic supporters to be uncritical of polarographic methods and to use a polarographic procedure whether or not some other more satisfactory method existed. Some of the polarographic analyses suggested in the literature are quite complex and do not compare favorably with simpler colorimetric, volumetric, and spectrophotometric methods available. A polarographic approach should be considered whenever new and more accurate methods of analysis for pharmaceuticals are being sought. For many pharmaceutical analytical problems, polarography may well offer a method that has advantages over all other techniques.

There are some official pharmaceutical preparations for which the present method of analysis does not provide for the separation of excipients prior to analysis. In some cases, the compound cannot be separated from its excipients by partition between immiscible solvents and there is no other convenient physical or chemical means of separation. These excipients may interfere with a particular assay and lead to erroneous results. This is especially a danger in an ultraviolet spectrophotometric assay. In some cases, excipients will not interfere with the polarographic analysis, for polarography will produce a wave which is specific for a given compound with an electroreducible or oxidizable group. Acetazolamide, chlorothiazide, and nitrofurantoin were chosen as examples.

EXPERIMENTAL

Apparatus

The polarographic waves were obtained with the Sargent model XXI recording polarograph. The cells used were either large (20-ml. sample volume) or small (3-ml. sample volume) H-type cells containing a saturated calomel electrode (S.C.E.) separated from the sample compartment by an agar plug and fritted-glass diaphragm. The electrode capillary delivered 1.136 mg. of mercury per second at a column height of 45 cm. with a drop-time of 4.20 seconds.

Received July 18, 1961, from the Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biological and Physical Sciences, Division of Pharmaceutical Chemistry, Washington 25, D. C.
Accepted for publication August 2, 1961.

Acetazolamide Tablets

Acetazolamide produces a polarographic wave having a half-wave potential ($E_{1/2}$) of -0.518 volts in 0.1 N hydrochloric acid solution *vs.* S.C.E. The diffusion current was found to be linear with concentration in the range between 8×10^{-5} M and 4×10^{-4} M.

Procedure.—Weigh and finely powder not less than 20 acetazolamide tablets. Weigh accurately a portion of the powder, equivalent to about 50 mg. of acetazolamide, transfer to a 100-ml. volumetric flask, and dissolve in about 40 ml. of boiling water. Heat the mixture on a steam bath for 15 minutes, cool to room temperature, add water to volume, and mix. Filter a portion of this solution, discarding the first 10 ml. of filtrate, and then pipet a 3-ml. aliquot of the filtrate, accurately measured, to a 25-ml. volumetric flask, add 2 ml. of 1 N hydrochloric acid, dilute to volume with water, and mix. Transfer a portion of this solution to an electrolytic cell, place in a constant temperature bath regulated at 25.0 ± 0.5° and remove the dissolved oxygen by aerating the solution with nitrogen for approximately 10 minutes. Insert the electrolytic cell under the capillary tip of the dropping mercury electrode assembly having a drop time of 4 to 5 seconds. Trace the polarogram from -0.20 to -0.75 volt vs. S.C.E. at a sensitivity which will give a suitable curve. Extrapolate the residual curve and draw parallel to it a line through the diffusion current plateau. Measure the height of the diffusion wave as the difference between the residual current and the diffusion current plateau. Calculate the quantity in mg. of $C_4H_6N_4O_3S_2$ in the portion of the tablets taken by the formula $C\,(I_{d\,\text{sample}}/I_{d\,\text{std.}})$ in which $I_{d\,\text{sample}}$ is the diffusion current value of the sample, C is the concentration of the standard solution, and $I_{d\,\text{std.}}$ is the diffusion current value of U.S.P. Acetazolamide Reference Standard determined similarly in a solution of the same composition containing about 60 mcg. in each ml.

Chlorothiazide Tablets

Chlorothiazide produces a polarographic wave having a half-wave potential ($E_{1/2}$) of -1.528 volts in an equimolar solution of 1 N ammonium chloride solution and 1 N ammonium hydroxide solution *vs.* S.C.E. The diffusion current was found to be linear

with concentration in the range between 1.2×10^{-4} M and 6×10^{-4} M.

Procedure.—Weigh and finely powder not less than 20 chlorothiazide tablets. Weigh accurately a portion of the powder equivalent to 100 mg. of chlorothiazide and transfer to a 100-ml. volumetric flask. Add 20 ml. of dimethylformamide, agitate for 5 minutes, and make to volume with an equimolar solution of 1 N ammonium chloride solution and 1 N ammonium hydroxide solution. Pipet a 3-ml. aliquot of this solution, measured accurately, to a 25-ml. volumetric flask and make to volume with distilled water. Transfer a portion of this solution to an electrolytic cell, place in a constant temperature bath regulated at 25.0 ± 0.5°, and remove the dissolved oxygen by aerating the solution with nitrogen for approximately 10 minutes. Insert the electrolytic cell under the capillary tip of the dropping mercury electrode assembly having a drop time of 4 to 5 seconds. Trace the polarogram from −1.25 to −1.75 volts *vs.* S.C.E. at a sensitivity which will give a suitable curve. Extrapolate the residual current curve and draw parallel to it a line through the diffusion current plateau. Measure the height of the diffusion wave as the difference between the residual current and the diffusion current plateau. Calculate the quantity, in mg., of $C_7H_6ClN_3O_4S_2$ in the portion of the tablets taken by the formula $C\,(I_{d\ \text{sample}}/I_{d\ \text{std.}})$, in which $I_{d\ \text{sample}}$ is the diffusion current value of the sample, C is the concentration of the standard solution, and $I_{d\ \text{std.}}$ is the diffusion current value of U.S.P. Chlorothiazide Reference Standard determined similarly in a solution of the same composition containing about 125 mcg. in each ml.

Nitrofurantoin Oral Suspension

Nitrofurantoin produces a polarographic wave having a half-wave potential ($E_{1/2}$) of −0.384 volt in an equimolar solution of 1 N ammonium chloride solution and 1 N ammonium hydroxide solution *vs.* S.C.E. The diffusion current was found to be linear with concentration in the range between 8×10^{-5} M and 4.8×10^{-4} M.

Procedure.—Pipet accurately an amount of nitrofurantoin oral suspension equivalent to approximately 50 mg. of nitrofurantoin into a 100-ml. volumetric flask. Add 20 ml. of dimethylformamide, agitate for 5 minutes, and make to volume with an equimolar solution of 1 N ammonium chloride solution and 1 N ammonium hydroxide solution. Pipet a 3-ml. aliquot of this solution, accurately measured, into a 25-ml. volumetric flask. Add 0.10 ml. of a 0.1% gelatin solution and make to volume with distilled water. Transfer a portion of this solution to an electrolytic cell, place in a constant temperature bath regulated at 25.0 ± 0.5°, and remove the dissolved oxygen by aerating the solution with nitrogen for approximately 10 minutes. Insert the electrolytic cell under the capillary tip of the dropping mercury electrode assembly having a drop time of 4–5 seconds. Trace the polarogram from −0.20 to −0.70 volt *vs.* S.C.E. at a sensitivity which will give a suitable curve. Extrapolate the residual current curve and draw a line parallel to it through the diffusion current plateau. Measure the height of the diffusion wave as the difference between the residual current and the diffusion current plateau. Calculate the quantity, in mg., of $C_8H_6N_4O_5$ in the portion of the suspension taken by the formula $C\,(I_{d\ \text{sample}}/I_{d\ \text{std.}})$, in which $I_{d\ \text{sample}}$ is the diffusion current value of the sample, C is the concentration of the standard solution, and $I_{d\ \text{std.}}$ is the diffusion current value of U.S.P. Nitrofurantoin Reference Standard determined similarly in a solution of the same composition containing about 60 mcg. in each ml.

Nitrofurantoin Tablets

Procedure.—Weigh and finely powder not less than 20 nitrofurantoin tablets. Weigh accurately a portion of the powder, equivalent to about 50 mg. of nitrofurantoin, and transfer to a 100-ml. volumetric flask. Proceed as directed in the assay for nitrofurantoin oral suspension, beginning with "add 20 ml. of dimethylformamide." Calculate the quantity in mg. of $C_8H_6N_4O_5$ in the portion of the tablets taken by the formula $C\,(I_{d\ \text{sample}}/I_{d\ \text{std.}})$, in which $I_{d\ \text{sample}}$ is the diffusion current value of the sample, C is the concentration of the standard solution, and $I_{d\ \text{std.}}$ is the diffusion current value of U.S.P. Nitrofurantoin Reference Standard determined similarly in a solution of the same composition containing about 60 mcg. in each ml.

DISCUSSION

The intersection-point method of measuring the diffusion current, as shown in Figs. 1, 2, and 3, was used for all polarographic waves. When the diffusion current so measured was plotted against concentration, a straight line was obtained (Fig. 4). Each point on the graph represents an average of three determinations. The experimental error by this method of analysis was found to be ±1%. It was felt that the greatest source of error in the analyses resulted from the measurement of the diffusion current height from the polarographic wave.

The results obtained by both polarography and U.S.P. XVI methods of analysis for each dosage form are listed in Table I. The table shows that

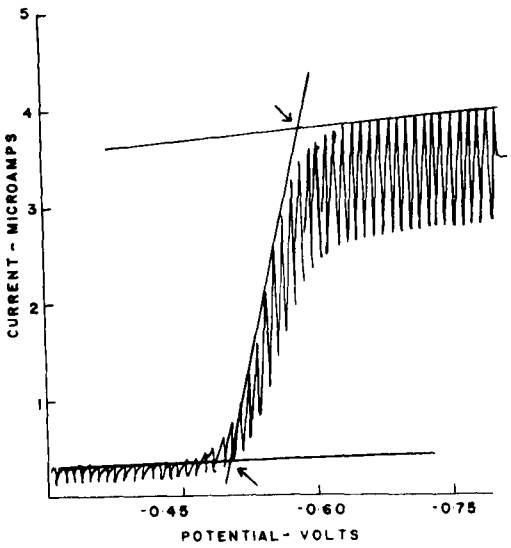


Fig. 1.—Acetazolamide polarogram in 0.1 N hydrochloric acid solution.

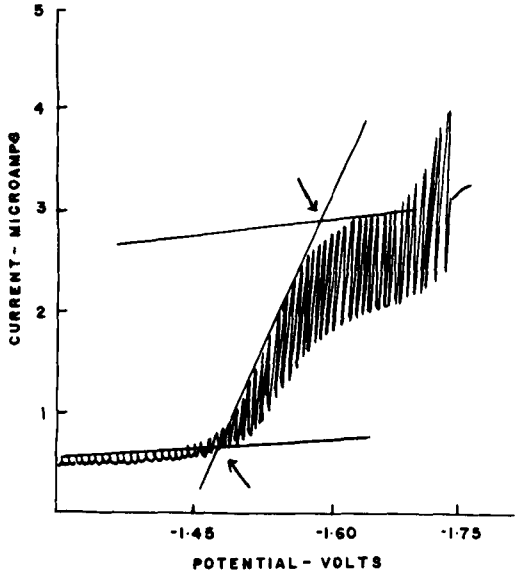


Fig. 2.—Chlorothiazide polarogram in equimolar ammonium chloride-ammonium hydroxide solution.

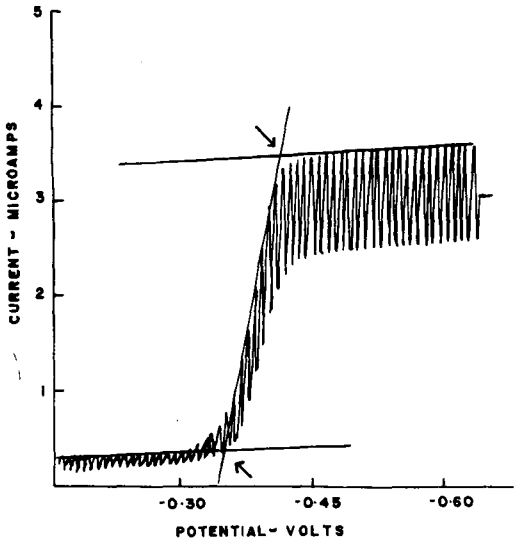


Fig. 3.—Nitrofurantoin polarogram in equimolar ammonium chloride-ammonium hydroxide solution.

the methods are in good agreement and that the results obtained by the polarographic method show greater precision than those obtained by the U.S.P. XVI method. In all cases, excipient material present in the commercially available preparations did not interfere with the polarographic and U.S.P. analysis and, therefore, no attempt was made to separate them prior to analysis. The average volume occupied by the undissolved tablet excipients was approximately 0.05 ml. so that the error introduced by leaving the tablet excipient in the solution is less

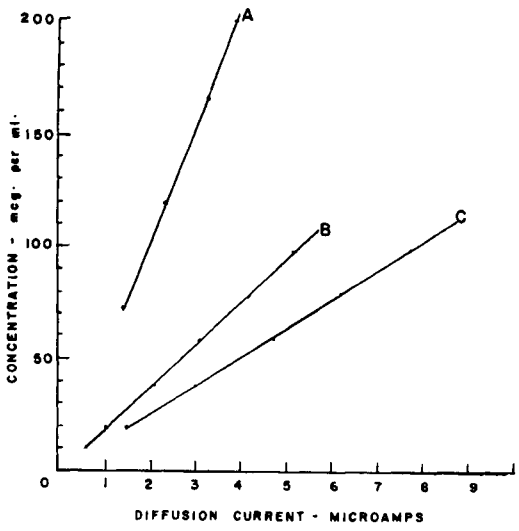


Fig. 4.—Plot of diffusion current *vs.* concentration for chlorothiazide (A), acetazolamide (B), and nitrofurantoin (C) solutions.

TABLE I.—RESULTS OF POLAROGRAPHIC AND U.S.P. XVI ANALYSES

Sample	% of Declared	
	Polarography	U.S.P. XVI
Acetazolamide tablets, 250 mg.	96.8	96.5
	96.8	97.2
	96.9	97.0
Chlorothiazide tablets, 25 mg.	102.4	101.3
	103.1	102.1
	103.3	...
	103.0	...
Nitrofurantoin tablets, 25 mg.	96.8	96.9
	96.6	96.1
	96.2	97.1
	95.7	96.3
Nitrofurantoin oral suspension	95.9	98.0
	96.3	97.4
	...	96.9[a]
	...	97.1[a]
Simulated nitrofurantoin oral suspension	99.2	99.4[a]
	99.2	99.7[a]

[a] U.S.P. XVI method modified by centrifuging sample after filtration.

than 0.2%, considerably less than the errors involved in measuring the diffusion current.

No difficulties were encountered with the polarographic analysis for acetazolamide, chlorothiazide, or nitrofurantoin in tablets and, therefore, outside of the development of a quantitative procedure no further investigation was undertaken. However, it was found that with the U.S.P. XVI spectrophotometric assay for nitrofurantoin oral suspension, a strong background was produced. This background can be attributed to the colloidal suspension produced by the Veegum present in the formulation which is not eliminated by simple filtration. The results obtained by the U.S.P. XVI method (Table I) for nitrofurantoin oral suspension are somewhat higher than those obtained by polarography. Modification of the U.S.P. XVI method by centrifuging the sample after filtration produced results which agreed more closely.

To determine whether the U.S.P. XVI or the polarographic method was at fault, a simulated nitrofurantoin oral suspension was prepared. Again, as shown in Table I, the polarographic and the modified U.S.P. XVI methods are in good agreement. It can be concluded that the spectrophotometric method is more susceptible to interference from extraneous materials than the polarographic method.

SUMMARY

Samples of acetazolamide, chlorothiazide, and nitrofurantoin were reduced at the dropping-mercury electrode. Their diffusion currents were found to be linear with concentration in the ranges investigated and therefore can be used for a quantitative polarographic assay.

A 0.1 *N* hydrochloric acid solution was found suitable for the polarographic determination of acetazolamide. A solution of dimethylformamide, 1 *N* ammonium chloride solution, and 1 *N* ammonium hydroxide solution were found suitable for the polarographic determination of both chlorothiazide and nitrofurantoin.

Similar experimental conditions were maintained throughout each assay to keep the variables at a minimum. The temperature was held within $\pm 0.5°$ by a constant temperature bath.

Polarographic analyses of dosage forms were compared with U.S.P. XVI analyses and proved to be quite satisfactory.

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Metal Chelates of Oxazolidinones as Central Nervous System Stimulants

By WINTHROP E. LANGE, BASIL H. CANDON, and MAX CHESSIN

In an attempt to determine the effect of chelation upon central nervous system activity, a series of metal chelates have been prepared of 2-imino-5-phenyl-4-oxazolidinone, 2-imino-5,5-diphenyl-4-oxazolidinone, and 2-imino-5-*p*-biphenyl-4-oxazolidinone, using Cu^{++}, Ni^{++}, Mg^{++}, and Fe^{+++} ions. Spectral and analytical evidence has indicated a 1:1 ratio of metal to oxazolidinone in the respective chelates of all four metals. The magnesium chelate of 2-imino-5-phenyl-4-oxazolidinone exhibited the selective central nervous system stimulating characteristics of the parent compound and it also provided two therapeutic advantages, an earlier onset of action and a relatively shorter span of activity.

In 1913 Traube (1) reported the synthesis of a series of oxazolidinones, but it was not until 1956 that Schmidt (2) called attention to the central stimulant activity of one of the compounds, 2-imino-5-phenyl-4-oxazolidinone. In 1957 Lienert and Janke (3) in a further study of the same compound showed an activity similar to one parameter of caffeine activity, namely central nervous system stimulation, without the objectionable side-effects of the alkaloid.

The presence of a center for chelation in the oxazolidinone molecule suggested that chelates could be prepared. The possibility then exists that metal chelates of an oxazolidinone might provide a water-insoluble compound with more desirable pharmacological activity. This then would be a start in a study of the effects of chelation on drugs which must cross the blood-brain barrier. Accordingly, various oxazolidinones were reacted with a number of metal salts capable of forming chelates, and several metal chelates have been prepared.

Received April 28, 1961, from the Massachusetts College of Pharmacy, Boston, and Nordson Pharmaceutical Laboratories, Inc., New York, N. Y.
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DISCUSSION

In order to determine the influence of substitution on the central nervous system stimulant activity of the oxazolidinone molecule, the following oxazolidinones were prepared.

To determine whether the U.S.P. XVI or the polarographic method was at fault, a simulated nitrofurantoin oral suspension was prepared. Again, as shown in Table I, the polarographic and the modified U.S.P. XVI methods are in good agreement. It can be concluded that the spectrophotometric method is more susceptible to interference from extraneous materials than the polarographic method.

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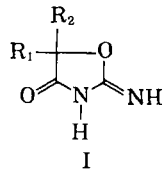
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In order to determine the influence of substitution on the central nervous system stimulant activity of the oxazolidinone molecule, the following oxazolidinones were prepared.



I

Ia. $R_1 = C_6H_5$, R_2 = H 2-imino-5-phenyl-4-oxazolidinone

Ib. $R_1 = C_6H_5$, $R_2 = C_6H_5$ 2-imino-5,5-diphenyl-4-oxazolidinone

Ic. $R_1 = C_6H_5—C_6H_5$, R_2 = H 2-imino-5-*p*-biphenyl-4-oxazolidinone

Chelates (1:1) with cupric, nickel, ferric, and magnesium ions were isolated and the analytical results are recorded in Table I. A stable 1:1 chelate with neither zinc nor ferrous iron could be obtained.

Structural Considerations.—The results of elemental analyses of the chelates, as shown in Table I, indicate a structure containing one metal atom per molecule of oxazolidinone. The presence of water of chelation was shown by a loss of weight corresponding to approximately the calculated number of molecules of water without a change in color when the chelates were dehydrated at 225°. In the case of the magnesium chelate, the additional molecule of water of hydration was determined by Karl Fischer titration without drying at 110° as was done with the other chelates. Saturated aqueous solutions of the nickel and copper chelates gave negative tests for the metal ions. A positive test was given by magnesium and ferric iron indicating the ionic character of these complexes.

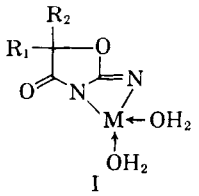
The infrared spectral characteristics of the oxazolidonones and their chelates were compared in an attempt to show the site of chelation. Owing to the complexity of the spectra of amide chelates in the 3 μ region, it is not suitable for detailed study. However, in the 6 μ region the intense amide I band can easily be identified. This band is usually considered to be a stretching wavelength of the amide carbonyl in resonance with the C—N band. In the oxazolidinone compounds the amide II band thought to be due to C—N stretching or N—H deformation is also present. Table II lists the wavelengths of the absorption bands for the various oxazolidinones and their chelates.

TABLE II.—INFRARED ABSORPTION BANDS, μ, OF OXAZOLIDINONE CHELATES[a]

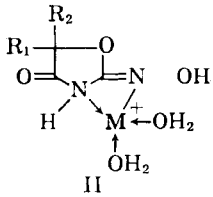
Compound	Bands		
	Amide I	Amide II	NH or OH
Ia	5.95	6.35[b]	7.73
Ia-Copper chelate	6.25	...	7.67
Ia-Nickel chelate	6.18	...	7.69
Ia-Magnesium chelate	6.00	6.35, 6.62	7.80
Ia-Ferric chelate	6.00	6.35, 6.42	7.75, 7.82
Ib	6.00	6.30	7.65
Ib-Copper chelate	6.10	...	7.70
Ic	6.00	6.38	7.80
Ic-Copper chelate	6.20	...	7.72

[a] The compounds were examined as solids in Nujol mulls using a Perkin-Elmer model 137 recording spectrophotometer. [b] A broad band extending to 6.65 μ.

It will be noted that in the case of the copper and nickel chelates the band corresponding to the amide I has a higher wavelength than the parent compound and the amide II band disappears. This could be due to a definite shift of the resonance equilibrium of the amide group and the formation of covalent bonds. The fact that there is no change in the intensity of the carbonyl or amide I band could indicate structure I as the most likely for the copper and nickel chelates.



I



II

In the case of the ferric and magnesium chelates, the amide I band remains unchanged while the amide II band shows two maxima. This could be due to coordination with the imino group giving rise to definite bands rather than the broad amide II band of the parent compound. These compounds then could have a structure corresponding to II which might indicate either a cyclic complex or a chelate. In general, chelate bonding of the alkaline earth ions is primarily ionic and the bond

TABLE I.—ANALYSES OF OXAZOLIDINONE METAL CHELATES[a]

Chelate[b]	Formula		C, %	H, %	N, %	M,[c] %	Water[d]	Color
Ia-$Mg^+OH^-\cdot 2H_2O$	$C_9H_{10}MgN_2O_4\cdot H_2O$	Calcd.	42.81	3.99	11.09	9.64	21.4	
		Found	42.0	4.3	9.9	10.4[e]	20.5[f]	white
Ia-$Mg^+OH^-\cdot H_2O$	$C_9H_9MgN_2O_4$	Calcd.	46.10	4.21	11.01	10.42	15.7	
		Found	45.85	4.0	12.2	10.45	15.8	white
Ia-$Cu\cdot 2H_2O$	$C_9H_{10}CuN_2O_4$	Calcd.	39.49	3.68	10.23	23.21	13.2	
		Found	40.9	3.6	9.9	22.3	12.7	blue
Ia-$Ni\cdot 4H_2O$	$C_9H_{14}N_2NiO_6$	Calcd.	35.44	4.63	9.18	19.24	23.6	
		Found	35.0	4.9	8.7	18.7	24.4	green
Ia-$Fe^{++}2OH^-\cdot 2H_2O$	$C_9H_{12}FeN_2O_6$	Calcd.	35.91	4.35	9.31	18.55	23.9	
		Found	35.5	3.9	8.9	19.2	25.0	brown
Ib-$Cu\cdot 2H_2O$	$C_{15}H_{14}CuN_2O_4$	Calcd.	51.47	4.03	8.0	18.17	10.3	
		Found	51.0	3.8	7.7	17.3	9.4	purple
Ic-$Cu\cdot 2H_2O$	$C_{15}H_{14}CuN_2O_4$	Calcd.	51.47	4.03	8.0	18.17	10.3	
		Found	51.6	3.8	7.8	17.5	10.9	blue

[a] The carbon-hydrogen analyses were determined by Weiler and Strauss, Oxford, England, and by Dr. C. K. Fitz, Needham, Mass. [b] Ia = 2-imino-5-phenyl-4-oxazolidinone; Ib = 2-imino-5,5-diphenyl-4-oxazolidinone; Ic = 2-imino-5-*p*-biphenyl-4-oxazolidinone. [c] The metal content of the chelates was determined by ashing to constant weight at 1000°. [d] This quantity is the loss of weight on dehydration at 225° after initial drying at 110°. [e] Determined gravimetrically as the pyrophosphate. [f] This sample was determined by the Karl Fischer method without drying at 110°.

strength is related to the ratio of charge of the ion to its ionic radius. The charge/radius ratio for magnesium is 2.44 which is at the lower limit for chelate formation. Thus, the relatively weak bonding of magnesium as compared with the strong bonding of copper accounts for the difference in the infrared spectra of the two chelates.

The band at 7.73 μ for 2-imino-5-phenyl-4-oxazolidinone, as may be seen in Table II, was found to undergo a regular shift from one chelate to another. It is interesting to note that the relative order of stability of the various divalent chelates was found to parallel the wavelength shifts of this band (Cu > Ni > Mg). The results agree with those obtained by Harkins and co-workers (4) with five membered chelate ring imidazole derivatives except for the position of the magnesium.

Examination of another physical property, the oil-water partition coefficient, was made not only to discover differences between the chelates and cyclic complexes or ionic chelates, but also to obtain information regarding the capabilities of the metal derivatives to penetrate the bloodbrain barrier. Oleyl alcohol was selected for this determination in accordance with the advantages ascribed by Albert (5). The coefficients were obtained by measuring spectrophotometrically the percentage of compound extracted by oleyl alcohol in the manner described by Foye and Duvall (6). Reference to Table III shows that the copper chelate is considerably more fat-soluble than any of the others. It will be noted in respect to biological applications that the increased liposolubility modifies the onset of action of these compounds. However, this effect does not appear to be the only determinant of central nervous system stimulant activity.

TABLE III.—OIL-WATER PARTITION COEFFICIENTS AT 25°

Compound	Concn., Aq. Phase × 10^4 M	pH Aq. Phase	Partition Coeff. Oleyl Alc./H_2O
Ia[a]	2.8	7.9	3.2
Ia-Magnesium chelate	2.1	8.9	4.3
Ia-Copper chelate	0.7	6.0	18.0
Ia-Nickel chelate	1.3	6.6	11.0

[a] Ia = 2-imino-5-phenyl-4-oxazolidinone.

Biological Results.—Initial tests on 2-imino-5-phenyl-4-oxazolidinone, known generically as pemoline, confirmed the findings of Schmidt (2). This compound induces central nervous system stimulation in various laboratory animals while the homologs described by Traube (1) were inactive. The results of preliminary pharmacological screening of related compounds and their chelates which we had prepared are given in Table IV. The compounds were screened in male albino mice (18–22 Gm.) for pharmacological activity. Attention was especially directed toward central nervous system stimulant action determined by visual observation. The compounds were tested at dose levels of 250 and 500 mg./Kg., p.o. It is apparent from the results shown in Table IV that substitution at position 5 is critical while chelation does not effect activity *per se*.

TABLE IV.—CNS STIMULANT ACTIVITY OF VARIOUS OXAZOLIDINONES AND THEIR CHELATES[a]

Compound[b]	No. of Mice	Dosage[c]	Activity[d]
Phenyl	20	250	A
	20	500	A
Phenyl, Cu	20	250	A
	20	500	A
Phenyl, Fe	20	250	A
	10	500	A
Phenyl, Mg	20	250	A
	20	500	A
Phenyl, Ni	20	500	A
Diphenyl	10	250	I
	20	500	I
Diphenyl, Cu	20	500	I
p-Biphenyl	20	250	I
	20	500	I
p-Biphenyl, Cu	20	500	I
Methyl	20	500	I
Diphenylene	10	250	I
	20	500	I
p-Bromophenyl	20	500	I
p-Hydroxy phenyl	20	500	I
p-Methoxy phenyl	20	500	I

[a] The results were obtained by visual observation. [b] The 5-position substituents of the 2-imino-4-oxazolidinone molecule is given and the metal when chelates are referred to. [c] The dosage is in mg./Kg., p.o. [d] A, active; I, inactive.

The relative lack of toxicity of magnesium in comparison with the other metals led to the selection of the magnesium chelate for further study. The compound induced central nervous system stimulation in monkeys, dogs, cats, rabbits, rats, and mice. Due to the insoluble nature of the compound, the oral route of administration was used throughout these studies except in rare instances. The duration and degree of central nervous system stimulatory activity in laboratory animals appears to be directly related to dosage. Although the acute oral LD_{50} value in mice was approximately 1500 mg./Kg. it was possible to note increased locomotor activity in doses as low as 15 mg./Kg. The profile of central nervous system stimulation noted does not resemble that induced by sympathomimetic amines, i.e., pilomotor erection, salivation, and widening of the palpebral fissures. Antihistiminic, cholinolytic, and adrenolytic effects were not observed in the pharmacological evaluation of this compound.

Unlike the monamine oxidase inhibitors, the serotonin and norepinephrine levels of the brains of the mice pretreated with magnesium pemoline did not differ from those of untreated control animals. Female albino mice weighing 18–24 Gm. were pretreated with magnesium pemoline (100 mg./Kg., p.o.) and sacrificed at scheduled times (1 hour, 3 hours, and 24 hours). Whole brains were removed and pooled. Serotonin and norepinephrine measurements were carried out by spectrophotofluorimetric methods (7, 8).

To measure the onset and duration of effect of the magnesium chelate compared to the base compound, screening studies were undertaken in mice, measuring the response to supramaxial electroshock by the methods of Toman and co-workers (9, 10).

Mice received supramaximal electroshock through cranial electrodes. The latency period was measured for convenience from onset of shock to onset of tonic extensor phase. It normally includes a

phase of increasing limb flexon. The threshold for tonic extensor convulsions is 7–10 ma. The latency period between shock and the extensor component of the convulsion was recorded to the nearest 0.2 sec. following 20 ma. (approximately twice the threshold shock).

After establishing that pemoline and magnesium pemoline significantly prolonged the supramaximal electroshock latency period following oral administration, each drug was administered orally to mice, generally in groups of 20. The dose of each compound was 100 mg./Kg., p.o. Treated groups and control groups were challenged at specified intervals and onset and duration of action were determined. Analysis of data presented in Table V reveals that the onset of activity of magnesium pemoline was more rapid. Statistically significant responses were obtained at 15 minutes while the parent compound was inactive at 15 minutes and 30 minutes, but was active at the 1-hour test period.

TABLE V.—ELECTROSHOCK SEIZURE LATENCY EXPERIMENTS

Expt. No.	Time of Electroshock Challenge after Drug, hr.[a]	No. of Mice/ Group	Increase in Seizure Latency Time Over Control Group Mg-Pemoline	Pemoline
1	1/4	10	S[b]	N.S
2	1/4	10	S	N.S
3	1/2	10	S	N.S
4	1/2	10	S	N.S
5	1	20	S	S
6	4	20	S	S
7	6	10	N.S	S
8	6	10	S	S
9	8	10	N.S	S
10	10	10	N.S	N.S
11	10	10	N.S	N.S

[a] The dosage used was 100 mg./Kg. orally. [b] S, Statistically significant increase in latency seizure time; N.S, not significant statistically (*t* test).

EXPERIMENTAL

2-Imino-5-phenyl-4-oxazolidinone.—This product was obtained by the procedure of Traube (1). A 83% yield of colorless needles was obtained which melted at 254–256° (decompn.) which agrees with the reported value.

2-Imino-5,5-diphenyl-4-oxazolidinone.—This compound was prepared by the procedure of Iwaya and workers (11). A white solid was obtained which melted at 250–252° (decompn.) which agrees with the reported value.

Ethyl-*p*-xenylglyoxalate.—This intermediate was prepared by the procedure of Blicke and Grier (12). Recrystallization of the product from an ether-petroleum ether mixture gave a white solid which melted at 38–39° which agrees with the reported value.

Ethyl-*p*-xenylhydroxyacetate.—In a pressure bottle was placed 25.4 Gm. (0.1 mole) of ethyl-*p*-xenylglyoxalate in 175 ml. of anhydrous ethanol, 0.85 Gm. platinized charcoal, and 6 ml. of water containing 0.35 Gm. of palladium chloride. The mixture was hydrogenated for 8 hours at three atmospheres pressure. The catalyst was removed by filtration and the solvent evaporated under reduced pressure. The residue was recrystallized from aqueous alcohol to give 23.0 Gm. (90%) of ethyl-*p*-xenylhydroxyacetate; m.p. 160–162°.

Anal.—Calcd. for $C_{16}H_{16}O_3$: C, 74.97; H, 6.29. Found: C, 74.81; H, 6.50.

2-Imino-5-*p*-biphenyl-4-oxazolidinone.—A solution of 12.8 Gm. (0.05 mole) of ethyl-*p*-xenylhydroxyacetate and 9.2 Gm. (0.05 mole) of guanidine nitrate in 50 ml. of ethanol and sodium ethoxide (1.5 Gm. sodium) was heated 30 minutes on a water bath. The ethanol was removed *in vacuo* and the residue dissolved in sodium bicarbonate. The aqueous solution was washed with ether and acidified with dilute hydrochloric acid to give 9.0 Gm. (70%) of 2-imino-5-*p*-biphenyl-4-oxazolidinone as a crystalline solid melting at 283–286° with decomposition.

Anal.—Calcd. for $C_{15}H_{12}N_2O_4$: C, 71.43; H, 4.79. Found: C, 71.11; H, 4.92.

Preparation of the Metal Chelates.—The chelates of the various oxazolidinones were all prepared by the same procedure. The metal salts which were employed were: cupric chloride, magnesium sulfate, ferric sulfate, nickel nitrate.

To a mixture of the oxazolidinone (0.025 mole) in 50 ml. of ethanol was added sufficient 20% sodium hydroxide solution to solubilize the oxazolidinone. The solution was diluted with 100 ml. of water and the metal salt (0.025 mole) in 20 ml. of water was slowly added with constant stirring. A sufficient quantity of 20% sodium hydroxide solution was added from time to time to maintain the pH at 10.5. The mixture was stirred for 1 hour after the final addition of the metal salt solution and the product collected by filtration. The yields of the various chelates ranged from 83 to 95% of the theoretical. Analyses and colors of the various chelates are shown in Table I.

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Manually Sorted Punched Card System for Pharmaceutical Literature

By JOHN G. WAGNER

A 5 × 8 inch punched card and direct coding system for pharmaceutical literature is illustrated and described. Use of the cards for a cooperative literature searching system and for a separate complete article file is discussed. Although the punched card system was designed for use in a department of product research and development in a pharmaceutical house, it would probably serve equally well for the faculty of a college of pharmacy.

A NUMBER of authors (1–9) have reported on the use of hand-sorted punched cards for bibliographic, data storage, and data processing purposes. Specific articles have been written on the use of such cards for a chemical bibliography (1), an instrumentation bibliography (7), storage of infrared absorption spectra (6), storage of medical research data (8), and processing of psychological data (9).

Keeping track of literature in the pharmaceutical field is becoming an increasing burden. It has become almost a necessity to have a cooperative effort with central filing of abstracts and/or references in order to be able to keep up with the new literature. This is true not only in industrial research and development but also in the academic field. The punched card system for pharmaceutical literature to be described was designed for use in a department of pharmacy or product research and development in a pharmaceutical house, but it would probably serve equally well for the faculty of a college of pharmacy.

The most useful property of punched cards is that of providing a means of cross indexing without the necessity of duplicating entries (4). In initiating the system to be described, most attention was paid to the facility in getting references and abstracts out of the file as in the system proposed by Cox, *et al.* (1).

THE PUNCHED CARD AND CODING SYSTEM

A 5 × 8 inch card sold by the Royal McBee Corporation (10) is used. Although the 155 holes around the perimeter of this card are divided into a series of numbered fields, the latter are not used in the system to be described. The numbers printed on the face of the card are completely ignored and only direct coding utilized, as shown in Fig. 1. This figure shows the back of the card. The material shown is printed directly on the cards in lots of one thousand by an offset printing process.

The "D" opposite a word such as Endocrine means deep punch, while the "S" opposite a word such as Agriculture means shallow punch. There are 58 pairs of "D" and "S" categories, making a total of 116 possibilities for direct coding. One additional hole is utilized if the reference is a patent or specification for a patent. Eight other holes near the cut corner of the card are used for the last two digits of the year; the last digit of the year of publication is placed in the set of four holes nearest the cut corner. The remaining 30 holes, 20 at one edge of the card and 10 at the other edge of the card, are numbered. The categories assigned to 29 of these 30 holes are shown in Table I.

In Fig. 1 there are several heavier black lines which divide the categories into several groups. Referring to the left-hand side of the back of the card, the groups of categories from top to bottom may be titled: pharmacologic classification, method of administration, type of dosage form, nature of process, assay and instrument classification, and source of reference. Referring to the right-hand side, the groups of categories from top to bottom may be titled: chemical and pharmaceutical nature of substance, stability and compatibility group, and a miscellaneous group which starts with "clinical testing" and ends with "drug combination(s)."

The cards may be filed by year and into "patent" and "nonpatent" sections. However, the cards could be filed randomly in one group or into any other specific groups. For example, if the card deck becomes large enough one might wish to segregate a group of categories, such as the assay and instrument classification.

A COOPERATIVE LITERATURE SEARCHING SYSTEM

Since we are now adding to the file of cards at the rate of about 1,200 cards per year, cooperative abstracting has been initiated in our department. Patents present no problem since we merely cut out pertinent abstracts of the patents from the *Official Gazette* and paste them on the cards. When a rubber cement is used a card will be perfectly flat after the abstract is pasted onto it. Most of the

Received September 6, 1961, from the Pharmacy Research Section, Product Research and Development Unit, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication November 3, 1961.

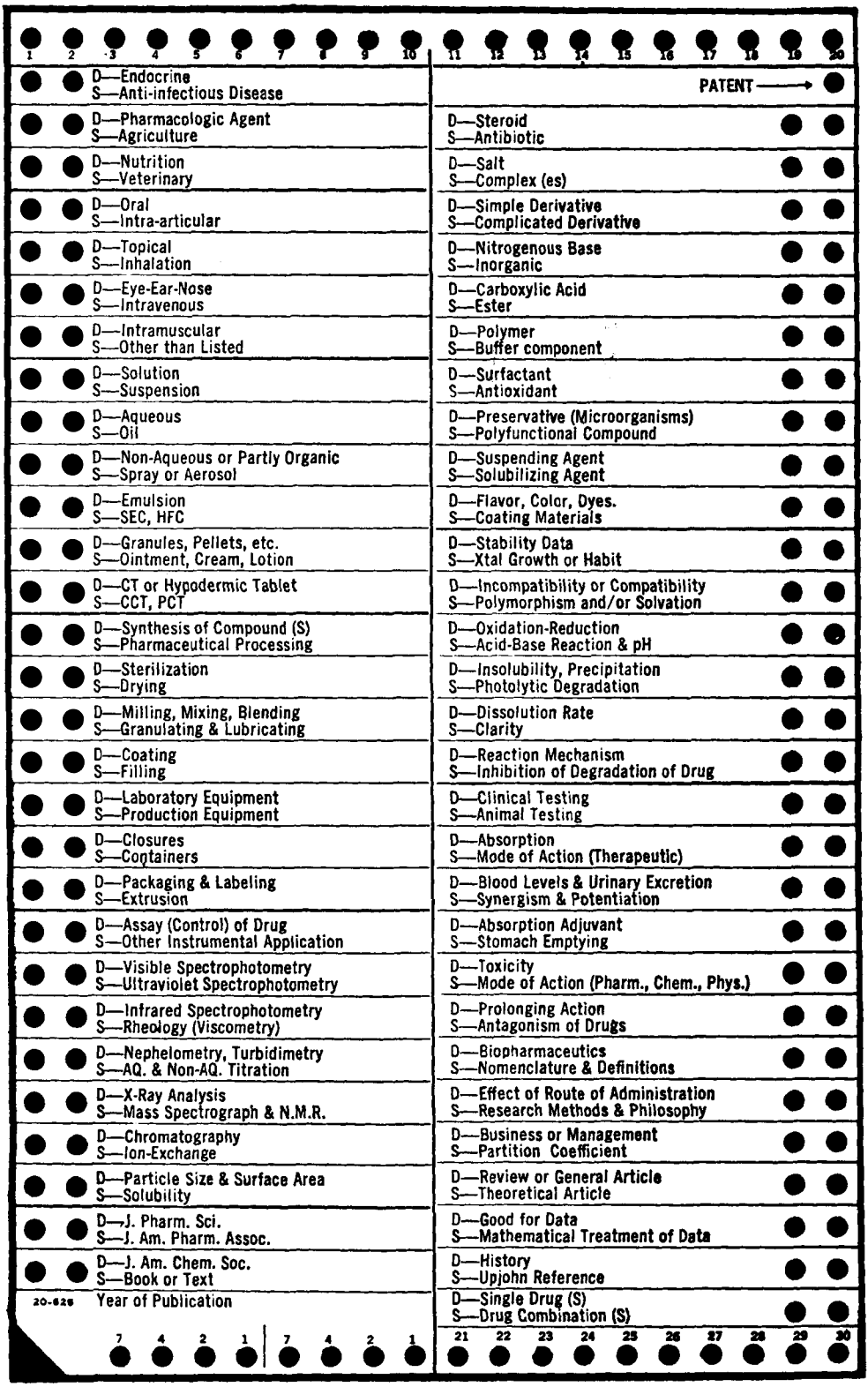


Fig. 1.—Copy of the direct coding system printed on the back of the 5 × 8 inch card.

TABLE I.—ADDITIONAL CATEGORIES FOR PUNCHED CARDS WHICH ARE NOT PRINTED ON THE BACK OF THE CARD

1. Adsorption
2. Colloidal phenomenon (phenomena)
3. Computers, tape input, programming, etc.
4. Correlation of chemical and physical properties with biological activity
5. Correlation of *in vitro* and *in vivo* rate data
6. Dental
7. Dielectric constant(s) and/or dipole moment(s)
8. Drug binding in physiological systems
9. Extensive bibliography
10. Government and government regulations
11. Graphical representation of data
12. Isomerism
13. Kinetics
14. Molecular and atomic structure
15. Molecular weight
16. Optical activity
17. Physical methods of measurement other than listed on card
18. Physiology
19. Proposals and ideas for future research and development
20. Report writing aids
21. Solvents for reactions
22. Solvents for recrystallizations
23. Stability constants
24. Statistics
25. Sustained action, oral
26. Sustained action, parenteral
27. Thermodynamic quantities
28. Weighing, automation of, etc.
29. Coating and fluidization

articles to be abstracted are chosen from *Current Contents* (11). The title of each article, the surnames and initials of the authors, and the journal reference is typed onto the front of the card. The cards are then assigned for abstracting and a simple record kept of the number of cards assigned to each scientist on a given day. When the cards are returned with the abstracts on them, an entry of the date they were returned is made and then the number of days required for abstracting is noted. When about 50 to 100 cards have been returned, the cards are coded using a china red marker to indicate where "D" or "S" cuts are to be made in the cards. Since only the abstractor would often know whether there are any categories involved in the numbered holes (Table I), the scientist doing the abstracting is requested to have Table I with him when he reads the article and to mark the appropriate holes with a china red marker.

The cards bearing abstracts are circulated to about fifteen scientists. Hence each scientist gets the benefit of the others' abstracting. When each deck of coded cards has completed the circuit of scientists, they are punched appropriately and filed in drawers.

RESULTS OF THE LITERATURE SEARCHING SYSTEM

The growth of the punched card file of pharmaceutical literature in the Product Research and Development Unit of The Upjohn Co. is shown in Fig. 2. The system was initiated on October 15, 1956. There has been steady growth in the number of cards added to the file in each subsequent year. At the present time the rate of addition is greater

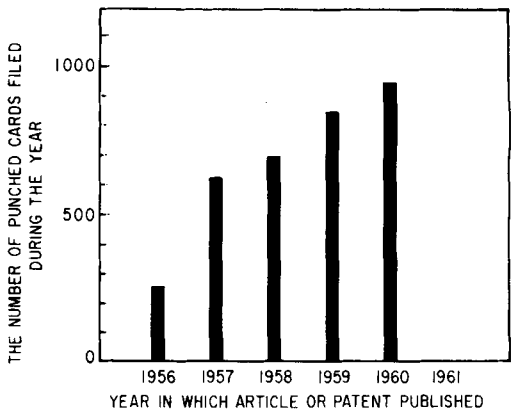


Fig. 2.—The growth of the punched card file of pharmaceutical literature in the Product Research and Development Unit of The Upjohn Co.

than 1,200 cards per year. It is estimated that by the end of 1962 there will be between 5,000 and 6,000 cards in the file.

The average time and range of time required for abstracting[1] articles varies widely, depending upon the particular scientist doing the abstracting and his duties at the time the cards are assigned. Average abstracting times for individual scientists range from 2 to 40 days with corresponding ranges from 1 to 7 days, and 13 to 72 days. During 1960 a total of 975 abstracted cards were circulated to 14 to 15 scientists. The average time for a deck of cards to circulate among the 14 or 15 scientists was 48 days, with a range of 16 to 82 days.

The usefulness of the file of punched cards may be illustrated by a few examples. When the author was requested to write a recent review article (12), it required only about 1 day to retrieve about 500 pertinent references and abstracts from the file. Reduction of these to about 200 references resulted in the principal material from which the review was written. When a scientist is assigned the task of writing an Invention Report for the Patent Law Unit, preparatory to the filing of a patent application, most of the pertinent patent and other literature references can be obtained readily from the file. The file has been used to answer some of the myriad of questions one is asked by personnel within and without his own unit of the Company during a working year; the file has even been used to answer telephone queries while the line has been held open.

A CARD SYSTEM FOR COMPLETE ARTICLE RETRIEVAL

The same type of card as shown in Fig. 1 may be used as an index to a complete article (reprint or photostat) file also. In this case only the title, author(s), reference, a code number, and the year of publication is placed on the front of the card. The code number is written large in the upper right-hand corner with the year of publication in brackets beneath the number. Reprints or photostats added to the file are numbered consecutively by attaching a red-bordered label to the upper right-hand corner of the first page of the article and stapling the label

[1] Time between assignment of the card and its return.

to the page in case the adhesive becomes weak in later years. The articles are filed consecutively by number. Each year's articles are placed in a separate hanging file in a conventional file drawer. The cards are coded after reading the article and are then filed. Retrieval of the articles via the card index has been found to be very rapid and efficient.

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Cellulose Acetate Succinate as an Enteric Coating for Some Compressed Tablets

By L. O. WILKEN, Jr., M. M. KOCHHAR, D. P. BENNETT, and F. P. COSGROVE

The preparation and testing of compressed tablets of barium sulfate and sodium chloride coated with cellulose acetate succinate (CAS) are described, and the statistical evaluation of the data obtained is presented. The most satisfactory coatings were obtained when the test material, in an acetone-ethyl acetate solution, was applied by a modified "pan" method using talc as a dusting powder. Generally, CAS coatings which were stable for 3 1/2 hours in Simulated Gastric Fluid USP XVI dissolved sooner than control coatings of CAP applied and treated in the same manner. Disintegration times were slightly lower for most tablets placed directly into Simulated Intestinal Fluid, USP XVI than they were for tablets first treated with Simulated Gastric Fluid USP XVI for 3 1/2 hours and then tested in the former solution. None of the tablet coatings showed signs of cracks as a result of storage at −2 ± 2° for 21 days or 45 ± 2° for 10 days; however, some of these showed statistically significant differences in average disintegration time when compared with samples stored at room temperature. When test and control tablets were stored at 40 ± 2° and approximately 81% humidity, the majority of coatings were unsatisfactory. Tablets lost less than 3.56% tablet weight as a result of the simulated gastric fluid treatment. Preliminary *in vivo* tests with human subjects indicate that CAS has merit as an enteric coating. Pancreatin appears to have little effect on the disintegration times of the test coating.

THE IMPORTANCE of a good enteric coating for compressed tablets has been long recognized (1–3). Recently, the interest in this area has been stimulated by reports (4–6) of new enteric coatings and improved methods of evaluating enteric coatings. While considerable studies have been reported on the use of cellulose acetate phthalate (CAP) (4, 7–9) as an enteric coating, little experimental work has been published on the use of cellulose acetate succinate (CAS) for this purpose. Malm, *et al.* (10), prepared some cellulose succinate compounds as early as 1940; however, the authors did not fully study the enteric coating properties of the compounds.

Since CAS and CAP appear to possess similar solubility properties, and since there still exists a need for more efficient and inexpensive enteric coating material, as well as more data on the substances presently employed for this purpose, this study was undertaken. Also, it was felt that CAS might be less toxic than corresponding phthalate products, if the former proved to be a satisfactory enteric coating material, since succinic acid is normally found in the body.

EXPERIMENTAL

Material.—The cellulose acetate phthalate and cellulose acetate succinate used were obtained from Eastman Kodak Co., Rochester, N. Y. CAS has the following composition (11): combined acetyl, 20.6%; combined succinyl, 33.6%; No. of acetyls per anhydroglucose unit, 1.65%; No. of succinyls per anhydroglucose unit, 1.15%; No. of hydroxyls per anhydroglucose unit, 0.20%; free succinic acid, 0.21%; free carboxyl (corrected for free succinic acid), 15.0%.

Received April 28, 1961, from the College of Pharmacy, University of Texas, Austin.
Accepted for publication July 18, 1961.
The authors wish to thank Distillation Products Industries for the generous samples of CAS and CAP used in this study.

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TABLE I.—PROPERTIES OF SOME PLAIN AND ENTERIC COATED TABLETS

Type of Tablet	Coats	Average Weight, Gm. ± S.D.	Punch Size and Shape, cm.	Av. Disintegration,[a] min. ± S.D.[b]	Average Thickness, cm. ± S.D.[b] Edges	Side
Plain Compressed Tablets						
NaCl 1	..	0.36 ± 0.01	0.949 (3/8 in.) deep concave	3 ± 0.6	0.954 ± 0.001	0.368 ± 0.006
NaCl 2n	..	0.37 ± 0.00	0.949 (3/8 in.) deep concave	3 ± 0.6	0.954 ± 0.002	0.373 ± 0.003
$BaSO_4$	..	0.46 ± 0.01	0.949 (3/8 in.) deep concave	5 ± 0.5	0.955 ± 0.001	0.386 ± 0.005
Enteric Coated Tablets (No Subcoats)						
CAS Talc NaCl 1	15	0.42 ± 0.01	...	12 ± 0.8	0.993 ± 0.003	0.389 ± 0.005
CAS Talc NaCl 2	15	0.44 ± 0.01	...	10 ± 0.5	1.000 ± 0.004	0.398 ± 0.007
CAP Talc NaCl 1	15	0.41 ± 0.01	...	25 ± 2.5	0.985 ± 0.003	0.386 ± 0.003
CAP Talc NaCl 2n	15	0.45 ± 0.01	...	28 ± 5.2	0.998 ± 0.006	0.410 ± 0.009
CAS Talc $BaSO_4$ 1	20	0.57 ± 0.01	...	19 ± 1.3	1.018 ± 0.004	0.440 ± 0.008
CAS Talc $BaSO_4$ 2	20	0.60 ± 0.01	...	24 ± 1.7	1.032 ± 0.005	0.461 ± 0.010
CAP Talc $BaSO_4$	20	0.58 ± 0.01	...	51 ± 0.9	1.022 ± 0.004	0.432 ± 0.007

[a] End point for all sodium chloride tablets was taken when 100% of tablet passed through screen of disintegration apparatus. End point for plain barium sulfate tablets was taken when the tablet edges showed definite erosion. [b] For disintegration times, standard deviation of a single observation of six tablets; for tablet dimensions, standard deviation of a single observation of 20 tablets.

TABLE II.—DISINTEGRATION TIMES OF TEST TABLETS

Enteric Coating	No. of Coats	Plain (P) Subcoated (SC)	Tablet Substance	Batch No.	Time, min. Exposed to Simulated Gastric Fluid U.S.P. XVI	Disintegration Time,[a] min. ± S. D. in Simulated Intestinal Fluid U.S.P. XVI, after Gastric Fluid Treatment	Disintegration Time,[a] min. ± S. D. in Simulated Intestinal Fluid U.S.P. XVI Only	Weight, Gm. ± S. D. of Tablet plus Enteric Coat	Weight, Gm. ± S. D. of Tablet or Weight, Gm. ± S. D. of Subcoated Tablet
CAS Talc	15	P	NaCl	1	210	12 ± 0.8	12 ± 0.7	0.42 ± 0.01	0.36 ± 0.01
CAS Talc	15	P	NaCl	2	210	10 ± 0.5	11 ± 0.9	0.44 ± 0.01	0.36 ± 0.00
CAP Talc	15	P	NaCl	1	210	25 ± 2.5	21 ± 0.6	0.41 ± 0.01	0.36 ± 0.01
CAP Talc	15	P	NaCl	2n	210	28 ± 5.2	28 ± 1.2	0.45 ± 0.01	0.37 ± 0.00
CAS Talc	20	SC	NaCl	1	210	28 ± 0.9	24 ± 1.2	0.95 ± 0.04	0.70 ± 0.01[b]
CAS Talc	20	SC	NaCl	2	210	27 ± 2.6	21 ± 1.1	0.92 ± 0.03	0.70 ± 0.01[b]
CAP Talc	20	SC	NaCl	..	210	49 ± 3.9	39 ± 1.9	0.96 ± 0.03	0.70 ± 0.01[b]
CAS Talc[c]	20	P	$BaSO_4$	1	210	19 ± 1.3	15 ± 0.8	0.57 ± 0.01	0.46 ± 0.01
CAS Talc	20	P	$BaSO_4$	2	210	24 ± 1.7	17 ± 1.1	0.60 ± 0.01	0.46 ± 0.01
CAP Talc	20	P	$BaSO_4$	..	210	51 ± 0.9	42 ± 2.3 (44 ± 4.6)	0.58 ± 0.01	0.46 ± 0.01
CAP Talc[c]	15	P	$BaSO_4$	..	210	29 ± 2.5	28 ± 0.9	0.53 ± 0.01	0.46 ± 0.01
CAS Talc	15	SC	$BaSO_4$	2	210	20 ± 1.2	17 ± 0.9	1.02 ± 0.06	0.85 ± 0.04
CAS Talc	20	SC	$BaSO_4$	1	210	23 ± 1.4	20 ± 1.0	1.02 ± 0.06	0.85 ± 0.04
CAS Talc[c]	15	SC	$BaSO_4$	1	210	16 ± 2.0	15 ± 0.8	Insufficient sample	0.85 ± 0.04
CAP Talc	20	SC	$BaSO_4$	..	210	32 ± 2.3 (35 ± 2.7)	27 ± 2.0 (33 ± 1.3)	1.03 ± 0.07	0.85 ± 0.04
CAP Talc[c]	15	SC	$BaSO_4$	..	210	29 ± 1.5	28 ± 2.4	Insufficient sample	0.85 ± 0.04

[a] End point for all sodium chloride tablets was taken when 100% of tablet passed through screen of disintegration apparatus. End point for subcoated barium sulfate tablets was taken at the first appearance of core tablet. End point for plain barium sulfate tablets was taken when the tablet edges showed definite erosion. [b] Weight and standard deviations of the average weights of 20 samples consisting of 10 tablets each. [c] Slight cracks observed in a few coatings after simulated gastric fluid treatment.

Other Materials Used.—Dimethyl phthalate (Eastman); ethyl acetate, N.F. and reagent; acetone, N.F. and technical; talc, U.S.P.; simulated gastric fluid U.S.P. XVI; simulated intestinal fluid U.S.P. XVI; sucrose U.S.P.; starch U.S.P.; calcium carbonate U.S.P.; amaranth U.S.P.; barium sulfate U.S.P., and sodium chloride, technical.

Preparation of Tablets.—Tablets of sodium chloride and barium sulfate-sucrose (5:2) were compressed on a power-driven Stokes model F single-punch machine using 3/8 in. deep concave punches. The weights of 10 tablets of sodium chloride and barium sulfate were maintained at approximately 3.60 and 4.60 Gm., respectively. The total weight of each granulation compressed was about 20 lb. Each batch was divided into two groups and designated as: (*a*) tablets to be enteric coated without a subcoat and (*b*) tablets to be subcoated prior to enteric coating. Simple syrup, subcoating powder (consisting of varying amounts of calcium carbonate, starch, and sucrose) and the conventional coating pan were used to apply the subcoats. For the barium sulfate tablets a small amount of amaranth, as an indicator, was added to some of

the subcoating powder and this mixture applied as the first two or three subcoats.

Tablets of each of the above two groups were then divided as follows: (*a*) tablets to be enteric coated without a dusting powder and (*b*) tablets to be enteric coated using talc as a dusting powder.

At a later date, another batch of sodium chloride (2n) was compressed as before, coated with CAP and talc, and used as a second control.

Enteric Coating.—The enteric coating solution used had the following composition: cellulose acetate succinate, 9.0 Gm.; dimethyl phthalate (plasticizer), 3.4 Gm.; ethyl acetate, 84.4 Gm.; acetone, 84.4 Gm. A solution containing the same concentration of commercially available CAP was used as the control.

The compressed tablets were enteric coated with the CAS or CAP solution using a modified "pan" coating technique. In place of the copper pan, a small porcelain container was used because the conventional pan was not suitable for small experimental lots of approximately 700 to 1500 tablets.

Twelve to fifteen milliliters of enteric coating solution were employed for each coat. The total number of coats applied per batch was 15 or 20. When a dusting powder was employed, approximately 12 to 15 Gm. of talc were used for each application. Controls were coated in a similar manner. It is estimated that the weight ratio of polymer to talc for tablets coated by this technique ranged from 1:10 to 1:15. Some properties of plain and enteric coated tablets are given in Tables I and II.

Enteric Coated Tablets.—*In vitro* tests were carried out by using the disintegration apparatus, simulated gastric fluid, and simulated intestinal fluid of the U.S.P. XVI.

Tablets coated only with 20 coats CAS or CAP did not resist the action of simulated gastric fluid U.S.P. XVI for $3^1/_2$ hours; therefore, they were rejected and not studied further.

All other enteric coated tablets were tested for disintegration in simulated intestinal fluid by two procedures: (*a*) by placing tablets directly into this fluid and (*b*) by placing the tablets in the simulated gastric fluid U.S.P. XVI for $3^1/_2$ hours prior to transferring them to the simulated intestinal fluid U.S.P. XVI. All disintegration times were recorded to the nearest minute.

The behavior of the tablets in the simulated gastric fluid, their disintegration times in simulated intestinal fluid, their end points for disintegration, and some of their physical properties are shown in Table II.

Effect of Storage.—Since it was observed by investigators that some enteric coatings may crack when subjected to various storage conditions, it was of interest to investigate the effect of cold, heat, and high humidity upon the test coatings. Eighteen to twenty-two tablets from each group of enteric coated tablets were exposed in open containers to: (*a*) refrigerator temperature of $-2 \pm 2°$ for 21 days, (*b*) oven temperature of $45 \pm 2°$ for 10 days, and (*c*) oven temperature of $40 \pm 2°$ and approximate humidity of 81%[1] for 10 days. At the end of the storage periods, tablets were allowed to return to room temperature, examined for cracks, weighed (except those rejected due to the action of high humidity chamber), and tested for disintegration as soon as possible. The condition in the humidity chamber was purposely made much more extreme than any which might be ordinarily found. With all sodium chloride test and control samples, a considerable amount of water was found in the storage containers and many of the coatings were broken. Most of the CAS and CAP coats on barium sulfate tablets containing no subcoats showed cracks; however, with the corresponding subcoated tablets, no breaks in the coatings were observed. These latter tablets increased in weight, but no further work was done with them. Test and control coatings stored in the heat and cold showed no cracks. The disintegration times for the samples are found in Table III.

Loss of Tablet Weight.—It was observed that many of the tablets gained weight during the simulated gastric fluid treatment and during the humidity storage period. This indicated that some liquid had entered into the tablets. Also, it was shown from the results of the humidity storage experiment that considerable sodium chloride was present in the water which had accumulated in the storage containers holding the nonsubcoated enteric coated sodium chloride tablets. These observations appear to substantiate Gakenheimer's (13) conclusion that CAP coatings may act as a semipermeable membrane. Therefore, it was of interest to determine whether the simulated gastric fluid caused any loss of tablet material during the testing procedure. Representative samples stored at room temperature, $-2 \pm 2°$ and $45 \pm 2°$ were weighed, placed in the simulated gastric fluid for $3^1/_2$ hours, and then dried to constant weight at $105 \pm 5°$. Corresponding samples not treated with the above solution but dried to constant weight at $105 \pm 5°$ served as controls. Considerable variation in tablet weight loss of both treated and control samples was observed. Results indicate, however, that not more than 3.56% tablet weight is lost as a result of the simulated gastric fluid treatment. Findings expressed as per cent weight loss of tablet material appear in Tables IV, V, and VI.

Effect of Different Solutions on Disintegration Times.—Bauer and Masucci (14) found that the disintegration of CAP coatings in the intestinal contents, which is on the acid side, is the result of the hydrolytic action of intestinal esterases and not upon the alkalinity of the solution. *In vitro* tests carried out in this study indicated that the per cent of pancreatin in the simulated intestinal fluid U.S.P. XVI had little effect upon the disintegration times of CAS or CAP coatings.

Findings suggest that if the pancreatic enzymes of the simulated intestinal fluid solubilize the coats by cleaving the ester linkages, the process is slow and overshadowed by the alkalinity of the solution. Results are given in Table VII.

In Vivo Test.[2]—Wagner (4) has pointed out that an adequate evaluation of an enteric coating necessitates both *in vitro* and *in vivo* tests. While a com-

[1] The proper humidity was maintained by placing in the bottom of a desiccator a saturated solution of ammonium sulfate containing an excess of the salt (12).

[2] We are indebted to Dr. J. H. Lawson, Consulting Radiologist, Health Center, University of Texas, for his help in evaluating the X-ray films.

TABLE III.—DISINTEGRATION TIMES OF TEST TABLETS FOLLOWING STORAGE

Enteric Coating	No. of Coats	Plain (P) Subcoated (SB)	Tablet Substance	Batch No.	Time, min. Exposed to Simulated Gastric Fluid U.S.P. XVI	Disintegration Time,[b] min. ± S.D.[c] in Simulated Intestinal Fluid U.S.P. XVI after Gastric Fluid Treatment and after Varied Storage Conditions: 45 ± 2° for 10 days	−2 ± 2° for 21 days
CAS Talc	15	P	NaCl	1	210	12 ± 1.1	12 ± 1.5
CAS Talc	15	P	NaCl	2	210	11 ± 0.2	10 ± 1.1
CAP Talc	15	P	NaCl	1	210	25 ± 2.8	24 ± 2.6
CAP Talc	15	P	NaCl	2n	210	30 ± 2.5	30 ± 1.5
CAS Talc	20	SC	NaCl	1	210	28 ± 2.1	24 ± 1.8
CAS Talc	20	SC	NaCl	2	210	27 ± 1.9	24 ± 1.4
CAP Talc	20	SC	NaCl	..	210	55 ± 4.2	45 ± 3.2
CAS Talc[a]	20	P	$BaSO_4$	1	210	13 ± 1.3	17 ± 0.6
CAS Talc	20	P	$BaSO_4$	2	210	22 ± 1.4	Not tested
CAP Talc	20	P	$BaSO_4$	..	210	53 ± 3.6	Not tested
CAP Talc[a]	15	P	$BaSO_4$	..	210	32 ± 1.3	31 ± 1.7
CAS Talc	15	SC	$BaSO_4$	2	210	18 ± 0.9	17 ± 1.3
CAS Talc	20	SC	$BaSO_4$	1	210	22 ± 1.5	20 ± 0.9
CAP Talc	20	SC	$BaSO_4$	..	210	33 ± 3.6	32 ± 2.0 34 ± 2.1
CAP Talc[a]	15	SC	$BaSO_4$	..	210	28 ± 1.5	24 ± 1.4

[a] Slight cracks observed in a few coatings after simulated gastric fluid treatment. [b] End point for all sodium chloride tablets was taken when 100% of tablet passed through screen of disintegration apparatus. End point for subcoated barium sulfate tablets was taken at the first appearance of core tablet. End point for plain barium sulfate tablets was taken when the tablet edges showed definite erosion. [c] Standard deviation of a single observation of disintegration times of six tablets.

TABLE IV.—LOSS OF WEIGHT DURING GASTRIC FLUID TREATMENT OF TABLETS STORED AT ROOM TEMPERATURE (CONTROLS)

Enteric Coating	No. of Coats	Plain (P) Subcoated (SC)	Tablet Substance	Batch No.	Weight Loss of Tablets Subjected to Gastric Fluid Treatment and 100 ± 5° Drying, %	Weight Loss of Tablets Subjected Only to 100 ± 5° Drying, %
CAS Talc	15	P	NaCl	1	3.57	0.397
CAS Talc	15	P	NaCl	2	3.08	0.579
CAP Talc	15	P	NaCl	1	1.62	0.605
CAS Talc	20	SC	NaCl	1	2.44	2.10
CAP Talc	20	SC	NaCl	..	3.15	2.71
CAS Talc	20	P	$BaSO_4$	2	2.38	1.27
CAP Talc	20	P	$BaSO_4$	..	1.15	1.01
CAS Talc	15	SC	$BaSO_4$	2	3.09	1.85
CAS Talc	20	SC	$BaSO_4$	1	2.26	2.09
CAS Talc	15	SC	$BaSO_4$	1	3.09	2.21
CAP Talc	20	SC	$BaSO_4$	..	2.80	1.73

TABLE V.—LOSS OF WEIGHT DURING GASTRIC FLUID TREATMENT OF TABLETS STORED AT 45 ± 2° FOR 10 DAYS

Enteric Coating	No. of Coats	Plain (P) Subcoated (SC)	Tablet Substance	Batch No.	Weight Loss of Tablets Subjected to Gastric Fluid Treatment and 100 ± 5° Drying, %	Weight Loss of Tablets Subjected Only to 100 ± 5° Drying, %	Weight Loss of Tablets During Storage at 45° for 10 Days, %
CAS Talc	15	P	NaCl	1	3.95	0.40	0.26
CAS Talc	15	P	NaCl	2	3.68	0.37	0.26
CAP Talc	15	P	NaCl	1	2.52	0.41	0.27
CAS Talc	20	SC	NaCl	1	1.13	1.06	1.16
CAS Talc	20	SC	NaCl	2	1.48	0.73	1.16
CAP Talc	20	SC	NaCl	..	0.94	1.15	1.16
CAS Talc	20	P	$BaSO_4$	1	1.46	0.90	0.79
CAS Talc	20	P	$BaSO_4$	2	0.97	0.67	0.59
CAP Talc	20	P	$BaSO_4$	..	0.88	0.87	0.39
CAS Talc	20	SC	$BaSO_4$	1	0.76	0.88	1.03
CAP Talc	20	SC	$BaSO_4$	..	0.93	1.07	0.88

plete *in vivo* study on CAS as an enteric coating was beyond the scope of this work, it was felt that a preliminary *in vivo* study on humans would be of interest to those working in the field of enteric coatings.

Two subjects were given CAS talc sc barium sulfate (20 coats) No. 1 tablets in the following manner: subject 1 fasted 6 hours before and during the experiment except for a cup of coffee at 10:00 a.m. A tablet was taken at 7:30 a.m. and again at 11:00 p.m. X-rays were taken at 11:05 a.m. and at 2:05 p.m. The radiologist's report states, "the 11:05 a.m. film shows an opacity resembling a medicinal coated tablet in the right lower quadrant which is probably in the ileum. On the 2:04 p.m. film fragmented density in the right lower quadrant indicates dissolution of the enteric coating with the material still in the ileum. A new tablet is observed in the right upper quadrant which is either in the distal end of the stomach or in the descending duodenum."

Subject 2 took food at 7:30 a.m. and at 12:15

TABLE VI.—LOSS OF WEIGHT DURING GASTRIC FLUID TREATMENT OF TABLETS STORED AT −2 ± 2° FOR 21 DAYS

Enteric Coating	No. of Coats	Plain (P) Subcoated (SC)	Tablet Substance	Batch No.	Weight Loss of Tablets Subjected to Gastric Fluid Treatment and 100 ± 5° Drying, %	Weight Loss of Tablets Subjected Only to 100 ± 5° Drying, %
CAS Talc	15	P	NaCl	2	2.30	0.579
CAP Talc	15	P	NaCl	1	2.35	0.605
CAP Talc	15	P	NaCl	2n	1.29	1.75
CAS Talc	20	SC	NaCl	2	2.60	2.19
CAP Talc	20	SC	NaCl	..	2.55	2.71
CAS Talc	20	P	$BaSO_4$	2	2.46	1.27
CAS Talc	15	SC	$BaSO_4$	2	2.90	1.85
CAS Talc	20	SC	$BaSO_4$	1	2.32	2.09
CAP Talc	20	SC	$BaSO_4$	..	1.88	1.73
CAP Talc	15	SC	$BaSO_4$	..	2.94	1.86

TABLE VII.—EFFECT OF DIFFERENT SOLUTIONS ON TABLET DISINTEGRATION TIME

Tablet	Coats	Solution	Disintegration Times, min. ± S. D.[a]
CAS Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI	17 ± 0.9
CAS Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI less pancreatin	19 ± 0.6 14 ± 0.6
CAS Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI less NaOH (pH 5.15)	No significant disintegration in 150 min.
CAP Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI	28 ± 2.4
CAP Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI less pancreatin	25 ± 3.8
CAP Talc subcoated $BaSO_4$	15	Simulated intestinal fluid U.S.P. XVI less NaOH	No significant disintegration in 150 min.

[a] Standard deviation of a single observation of disintegration times of six tablets.

p.m., only one tablet was taken at 8:00 a.m. X-rays were taken at 11:38 a.m. and at 2:10 p.m. The radiologist reported, "that both films show an undissolved medicinal coated tablet in the stomach."

A third subject also took food at breakfast and lunch. This subject received three CAS talc sc barium sulfate (15 coats) No. 2 tablets in the following way: one tablet was taken at 7:45 a.m., another at 10:00 a.m., and the third at 12:15 p.m. Only one X-ray was taken at 2:05 p.m. The results reported by the radiologist states, "A fairly homogeneous opaque material is noted in the caecum on the film. There is no indication of separation of the opaque material and this could be one or three tablets. There is one density at the top of the film overlying the spine which appears to be a tablet in the stomach, however, the stomach is not sufficiently included on the film for this determination to be definite."

Evaluation of Results.—Statistical evaluation of the results was performed using the *t*-test for determination of significant differences between two means. The *t* values that are greater than 95% probability *t* value at the appropriate degrees of freedom are considered significant. Results are given in Table VIII.

DISCUSSION

Generally, duplicate batches of each type of tablet were enteric coated and tested in an attempt to obtain reliable results.

Measurements of tablet thickness on the edge and side were made only for plain sodium chloride; plain barium sulfate; nonsubcoated, enteric coated sodium chloride; and nonsubcoated, enteric coated barium sulfate tablets because it was difficult to achieve satisfactory measurements with the subcoated, enteric coated tablets. Although the enteric coats of the latter tablets were quite evenly applied, the subcoats were not completely smooth. However, the weights of subcoats and enteric coats of these tablets were fairly uniform, and the tablets in general had an acceptable appearance. Overcoming the coating inconsistencies appeared to be a matter of developing a better coating technique.

While an attempt was made during the coating procedure to keep constant the volumes of enteric coating solution, the weight of dusting powder, the time between the addition of enteric coating solutions and the addition of talc, and the drying time between coats, it was noted that the movement of the tablets in the coating container had an effect upon the quantity of talc adhering to the tablets per application. When tablets were mixed frequently by hand while they were in the coating container, it appeared that less dusting powder adhered to the coatings. Two batches were rejected because it was felt they were not coated correctly. With one of these, the coatings contained some clear spots where little or no talc appeared to be present. The weight and instability of CAS talc plain barium sulfate No. 1 tablets in simulated gastric fluid appears to substantiate this observation. While this batch received the same number of coats as CAS talc plain barium sulfate No. 2, there was less enteric coating material on the tablets. A minimum amount of tablet stirring should be done during the coating procedure in order to prevent erosion of talc from the coatings. Wagner, *et al.* (4), has reported the importance of the talc concentration in three modern enteric

TABLE VIII.—EVALUATION OF TABLET DISINTEGRATION TIMES DATA AFTER VARIED STORAGE CONDITIONS

Enteric Coating	No. of Coats	Plain (P) Subcoated (SC)	Tablet Substance	Batch No.	RT 45°/10 days (H) −2°/21 days (C)	Standard Error	Degrees of Freedom	t Calcd.[a]	t at 95% Level
CAS Talc	15	P	NaCl	1	RT	0.33			
CAP Talc	15	P	NaCl	1	RT	1.0	10	12.1	2.228
CAS Talc	20	SC	NaCl	1	RT	0.37			
CAP Talc	20	SC	NaCl	1	RT	1.6	10	12.8	2.228
CAS Talc	20	SC	$BaSO_4$	1	RT	0.57			
CAP Talc	20	SC	$BaSO_4$	..	RT	0.92	9	8.33	2.262
CAS Talc	15	P	NaCl	1	C	0.61			
CAP Talc	15	P	NaCl	1	C	1.2	9	8.93	2.262
CAS Talc	20	SC	NaCl	1	C	0.74			
CAP Talc	20	SC	NaCl	1	C	1.4	10	13.3	2.228
CAS Talc	20	SC	$BaSO_4$	1	C	0.37			
CAP Talc	20	SC	$BaSO_4$	..	C	0.82	10	15.6	2.228
CAS Talc	15	P	NaCl	1	H	0.45			
CAP Talc	15	P	NaCl	1	H	1.1	10	10.8	2.228
CAS Talc	20	SC	NaCl	1	H	0.86			
CAP Talc	20	SC	NaCl	1	H	1.7	10	14.1	2.228
CAS Talc	20	SC	$BaSO_4$	1	H	0.61			
CAP Talc	20	SC	$BaSO_4$	1	H	1.0	10	9.4	2.228
CAS Talc	15	P	NaCl	1	RT	0.33			
CAS Talc	15	P	NaCl	1	C	0.61	10	0.0	2.228
CAS Talc	15	P	NaCl	1	RT	0.33			
CAS Talc	15	P	NaCl	1	H	0.45	10	00.0	2.228
CAS Talc	20	SC	NaCl	1	RT	0.37			
CAS Talc	20	SC	NaCl	1	C	0.74	10	4.84	2.228
CAS Talc	20	SC	NaCl	1	RT	0.37			
CAS Talc	20	SC	NaCl	1	H	0.86	10	0.0	2.228
CAP Talc	15	P	NaCl	1	RT	1.0			
CAP Talc	15	P	NaCl	1	C	1.2	9	0.64	2.262
CAP Talc	15	P	NaCl	1	RT	1.0			
CAP Talc	15	P	NaCl	1	H	1.1	10	0.0	2.228
CAP Talc	20	SC	NaCl	1	RT	1.6			
CAP Talc	20	SC	NaCl	1	C	1.4	10	1.89	2.228
CAP Talc	20	SC	NaCl	1	RT	1.6			
CAP Talc	20	SC	NaCl	1	H	1.7	10	2.02	2.228
CAS Talc	20	P	$BaSO_4$	1	RT	0.53			
CAS Talc	20	P	$BaSO_4$	1	C	0.25	10	3.42	2.228
CAS Talc	20	P	$BaSO_4$	1	RT	0.53			
CAS Talc	20	P	$BaSO_4$	1	H	0.53	10	8.0	2.228
CAP Talc	15	P	$BaSO_4$	..	RT	0.37			
CAP Talc	15	P	$BaSO_4$	..	C	0.69	10	2.55	2.228
CAP Talc	15	P	$BaSO_4$	..	RT	0.37			
CAP Talc	15	P	$BaSO_4$	..	H	0.53	10	4.62	2.228
CAP Talc	20	P	$BaSO_4$	..	RT	0.69			
CAP Talc	20	P	$BaSO_4$	..	H	1.0	10	1.64	2.228
CAP Talc	20	SC	$BaSO_4$	..	RT	0.92			
CAP Talc	20	SC	$BaSO_4$	..	C	0.82	10	0.81	2.228
CAP Talc	20	SC	$BaSO_4$	..	RT	0.92			
CAP Talc	20	SC	$BaSO_4$	..	H	1.0	10	0.74	2.228
CAS Talc	20	SC	$BaSO_4$	1	RT	0.57			
CAS Talc	20	SC	$BaSO_4$	1	C	0.37	10	4.42	2.228
CAS Talc	20	SC	$BaSO_4$	1	RT	0.57			
CAS Talc	20	SC	$BaSO_4$	1	H	0.61	10	1.2	2.228

[a] t was calculated by $t = \frac{|X_1 - X_2|}{\sqrt{(S\bar{x}_1)^2 + (S\bar{x}_2)^2}}$ as found in Martin, E. W., and Cook, E. F., "Remington's Practice of Pharmacy," 12th ed., Mack Publishing Company, Easton, Pa., 1961, pp. 109–110.

coatings. Sheffield, *et al.* (15), also, found some differences between tablets enteric coated in small lots and those enteric coated in pilot-size batches. These differences might be reflected in the amount of extra rubbing caused by the increase in number of tablets used.

While high humidity affected most of the coatings, this problem does not appear to be a serious one. Water-proof coatings can be applied to either the plain or finished tablet without too much difficulty. A desiccant can also be packaged with the finished product.

Some test and control tablets stored in the cold and heat showed significant changes in disintegration times when compared statistically with those kept at room temperature; however, it is felt that the differences were not too serious from a practical aspect. It should be noted that some water could have come in contact with the tablets stored at $-2 \pm 2°$ for 21 days inasmuch as they were stored in open containers directly under the freezing unit of the refrigerator.

Significant differences in disintegration times in simulated intestinal fluid were observed between CAS and CAP coated tablets. These results might be due to the differences in the chemical structure

of the two coating materials. Hiatt (16) stated that those cellulose acetate phthalates having a free carboxyl content between 9 and 15% by weight are most preferable for enteric coating. The CAS used in this report had a free carboxyl content of 15% by weight.

Wagner, *et al.* (17), also reported that the minimum pH at which starch acetate phthalates dissolve depends upon the percentage of free carboxyl groups on the polymer molecule.

Our findings on the disintegration times for tablets in simulated intestinal fluid following treatment in simulated gastric fluid, as compared with disintegration times for tablets placed directly into the former solution, generally agree with data reported by Wagner, *et al.* (4). Further work on this phase of the problem will be considered at a later date.

Pancreatic enzymes may cleave the ester linkages of CAS; however, this was not demonstrated in this study chiefly because of the relatively high pH of the solutions used. Further work using buffer solutions at pH values from 1.2 to 6.9 might indicate the pH dependency and stability of CAS.

Wagner and Long (4) point out that an adequate *in vitro* comparison of two or more enteric coatings as done here with CAP and CAS requires testing the tablets coated with the enteric material at several levels. The slopes and positions of lines obtained by plotting disintegration times against mean weight of coating per tablet or mean weight of enteric substance per tablet should then be compared. These investigators also state that the amounts of enteric material and dusting powder employed are equally as important as the nature of the enteric substance when comparisons are made.

In this study the two different coatings were compared only at two levels of enteric substance; however, an attempt was made to control the amount of talc applied so that test and control tablets would receive the same amount of dusting powder per tablet. Our results, at the levels tested, showed that CAS coatings dissolved faster than CAP coatings in simulated intestinal fluid; however, it is possible in light of the work of Wagner, *et al.* (4), at higher or lower levels of coating, the reverse might take place.

Preliminary *in vivo* tests show that CAS has merit as an enteric coating. While considerable *in vivo* testing is still required before definite conclusions can be drawn, it is of interest to note that with one test subject a CAS coated tablet remained in the stomach for a period of more than 6 hours. X-ray films of two other test subjects indicated that the CAS coated tablets dissolved shortly after entering the intestines.

SUMMARY AND CONCLUSIONS

1. The preparation and testing of cellulose acetate succinate coated tablets of sodium chloride and barium sulfate are described and the statistical evaluation of the data obtained is presented.

2. Satisfactory enteric coated tablets were obtained, in most instances, when CAS, in an acetone-ethyl acetate solution, was applied by a modified "pan" method using talc as a dusting powder.

3. None of the test coatings showed signs of cracks as a result of storage at $-2 \pm 2°$ for 21 days or at $45 \pm 2°$ for 10 days; however, some test and control coatings showed differences in disintegration times in simulated intestinal fluid U.S.P. XVI when compared statistically with samples kept at room temperature.

4. CAS coatings which were stable for $3^1/_2$ hours in simulated gastric fluid dissolved sooner than control coatings of CAP applied and treated in the same manner.

5. Disintegration times are slightly lower for most tablets placed directly into simulated intestinal fluid than for tablets first treated with simulated gastric fluid for $3^1/_2$ hours and then tested in the former solution.

6. Of the tablets tested, none lost more than 3.56% tablet weight as a result of the $3^1/_2$ hours treatment in simulated gastric fluid.

7. The presence of pancreatin in the simulated intestinal fluid appeared to have little effect upon the rate at which the coatings dissolved.

8. Preliminary *in vivo* tests with human subjects indicate that CAS has merit as an enteric coating.

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Qualitative and Quantitative Tests for Mepivacaine Hydrochloride

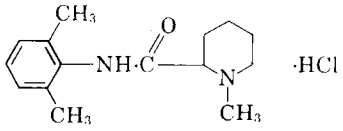
By M. E. AUERBACH, J. J. HEFFERREN, H. M. KOEHLER, and E. L. PRATT

A series of qualitative tests and quantitative methods are presented for mepivacaine hydrochloride. These data result from a cooperative laboratory study by research personnel of the drug firm and the American Dental Association. The methods chosen are discussed, their particular applications noted, and their limitations described.

MEPIVACAINE HYDROCHLORIDE[1] is a local anesthetic agent which is used in dentistry alone or with a vasoconstrictor such as levonordefrin (1). The chemical tests have been developed to provide data on those drugs appearing in "Accepted Dental Remedies." The limits set for these tests seem to be reasonable with expected normal analytical and manufacturing variation for mepivacaine hydrochloride and its preparations. It is to be expected that there may be or will be dosage forms of mepivacaine hydrochloride where these tests will not apply without appropriate modifications.

MEPIVACAINE HYDROCHLORIDE

1-Methyl-2,6-pipecoloxylidide hydrochloride; N-(2,6-dimethylphenyl)-1-methylpiperidine-2-carboxamide hydrochloride; $C_{15}H_{22}N_2O \cdot HCl$; mol. wt. 282.82. The structural formula of mepivacaine hydrochloride may be represented as follows



Physical Properties.—Mepivacaine hydrochloride is a white, odorless, crystalline solid, m.p. 255–262° (decompn.), U.S.P. class 1. It is freely soluble in methanol and water, very slightly soluble in chloroform, and practically insoluble in ether. The pH of a 2% aqueous solution is about 4.6.

Identity Tests.—Dissolve about 100 mg. of mepivacaine hydrochloride in 10 ml. of water and add 2 drops of nitric acid, followed by 1 ml. of silver nitrate T.S.; a white precipitate forms. This precipitate is insoluble in diluted nitric acid but soluble in diluted ammonia solution (presence of chloride).

Dissolve about 100 mg. of mepivacaine hydrochloride in 10 ml. of water, heat almost to the boiling point, and add with stirring 1 ml. of a saturated solution of picric acid in 20% alcohol. Collect the precipitate and wash with a few small portions of water and dry. The picrate melts with decomposition at 197–199°, U.S.P. class I. (Caution! Picrates may be explosive.)

Dissolve about 150 mg. of mepivacaine hydrochloride in 10 ml. of water, and transfer to a small separator. Add 10 ml. of chloroform and 3 ml. of ammonium hydroxide T.S. Shake the separator, allow the layers to separate, and carefully transfer the chloroform layer to a small flask containing 0.5 Gm. of sodium sulfate. Shake the contents until the chloroform solution is clear. Filter the solution and collect the filtrate in a small beaker. Evaporate the chloroform and dry the residue at 60° in vacuum. The yellow-white mepivacaine base melts at 149–153°, U.S.P. class I.

Absorption Characteristics.—A 0.03% solution of mepivacaine hydrochloride in water exhibits ultraviolet absorbance maxima at about 263 and 271 mμ [absorptivity (1%, 1 cm.) about 16.5 and 13.4, respectively] and minima at 254 and 270 mμ.

The infrared absorption spectrum of a 0.3% mixture of mepivacaine hydrochloride in potassium bromide, in a disk of about 0.83 mm. thickness, is shown in Fig. 1.

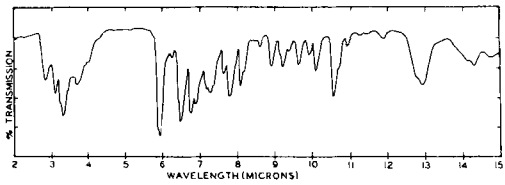


Fig. 1.—Infrared absorption of mepivacaine hydrochloride.

Purity Tests.—Dry about 1 Gm. of mepivacaine hydrochloride, accurately weighed, at 105° for 4 hours: the loss in weight does not exceed 1.0%.

Char about 1 Gm. of mepivacaine hydrochloride, accurately weighed, cool the residue, add 1 ml. of concentrated sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh. The residue does not exceed 0.1%.

Assay.—Transfer to a 200-ml. tall form beaker about 350 mg. of mepivacaine hydrochloride, ac-

Received January 8, 1962, from the Division of Chemistry, American Dental Association, Chicago, Ill., and Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Accepted for publication January 17, 1962.

Analysis of Local Anesthetics VII. For the preceding paper in this series, see THIS JOURNAL, 50, 347(1961).

[1] The trade name for mepivacaine hydrochloride is Carbocaine Hydrochloride.

curately weighed. Dissolve in 50 ml. of glacial acetic acid and add 10 ml. of a 6% solution of mercuric acetate in glacial acetic acid. Titrate potentiometrically with 0.1 *N* perchloric acid in glacial acetic acid, or titrate to a green end point using 2 drops of crystal violet in glacial acetic acid as an indicator. A blank should be run on all reagents. Each milliliter of 0.1 *N* perchloric acid is equivalent to 28.28 mg. of mepivacaine hydrochloride. The amount of mepivacaine hydrochloride, $C_{15}H_{22}N_2O \cdot HCl$, is not less than 98.0 nor more than 102.0%.

MEPIVACAINE HYDROCHLORIDE AND LEVONORDEFRIN INJECTION

Identity Tests.—The solution responds to the identity tests for mepivacaine hydrochloride.

Assay.—*Mepivacaine Hydrochloride.*—Transfer to a 60-ml. separator an amount of solution, accurately measured, equivalent to about 40 mg. of mepivacaine hydrochloride. Add 8 ml. of water and 1 ml. of 1 *N* sodium hydroxide and extract the mixture with three 10-ml. portions of chloroform. Wash the combined chloroform extracts with 10 ml. of water. Filter the chloroform extracts through a tight cotton plug, using additional chloroform to complete the transfer. Titrate metachromatically to a red end point with 0.01 *N* perchloric acid in dioxane, using 2 drops of 0.1% methyl red in methanol as an indicator. Alternately, add sufficient acetonitrile to the combined, washed chloroform extracts to give a chloroform-acetonitrile ratio of about 4 to 1 and titrate potentiometrically. Each milliliter of 0.01 *N* perchloric acid is equivalent to 2.828 mg. of mepivacaine hydrochloride. The amount of mepivacaine hydrochloride, $C_{15}H_{22}N_2O \cdot HCl$, is not less than 95.0 nor more than 105.0% of the labeled amount.

Levonordefrin.—The concentration of levonordefrin may be determined using the U.S.P. colorimetric assay procedure for epinephrine (U.S.P. XVI, p. 895). When the ferro-citrate solution and the glycine-bicarbonate buffer are mixed with a solution containing levonordefrin and mepivacaine hydrochloride, a fine precipitate forms. This precipitate should be removed by centrifuging before the colorimetric measurements of the iron complex with levonordefrin at 530 mμ are taken. This precipitate does not interfere with the reaction. The amount of levonordefrin is not less than 95.0 nor more than 110.0% of the labeled amount.

DISCUSSION

U.S.P. terminology for solubility, melting points, and reagents have been used wherever feasible.

Identity Tests.—The paper chromatographic R_f values for mepivacaine hydrochloride in hydrochloric acid and acetic acid systems described by Koehler and Feldmann (2) are 0.69 and 0.81, respectively.

Mepivacaine hydrochloride readily forms a reineckate salt in aqueous solutions. Tetraphenyl borate of mepivacaine hydrochloride forms with difficulty, thus it is not included in the identity caine base reacts with cobalt chloride T.S. to give a green color and a fine precipitate (3). A negative test with Millon's mercuric nitrate reagent can be used to determine absence of local anesthetics derived from *p*-aminobenzoic acid or other compounds with a primary amino group. The diazotation reaction with sodium nitrite and hydrochloric acid followed by a coupling reaction with beta-naphthol can be used to determine the presence of any impurities which have a primary aminophenyl group.

Quantitative Methods.—Mepivacaine hydrochloride, being the hydrochloride salt of a basic cyclic tertiary amine, can be determined by a number of the usual methods such as the Kjeldahl nitrogen, volumetric, or other chloride methods, the titration in a water-alcohol or nonaqueous medium of the amine base which has been extracted from an alkaline solution, and the direct perchloric acid colorimetric or potentiometric titration of the hydrochloride salt in a mixture of glacial acetic acid and mercuric nitrate, Fig. 2. This last method does not require a standard and offers a convenient, precise (standard deviation, 0.2%) method for the determination of the active ingredient, mepivacaine hydrochloride.

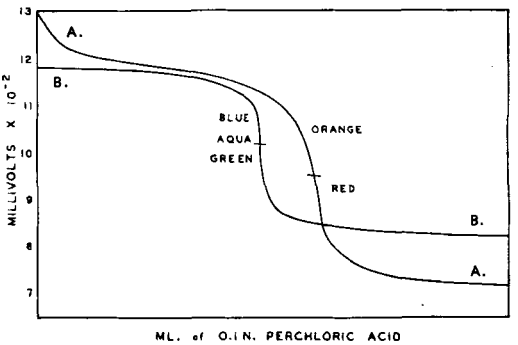


Fig. 2.—Potentiometric titration of mepivacaine base in a 4:1 mixture of chloroform-acetonitrile with 0.01 *N* perchloric acid in dioxane as a titrant and methyl red as an indicator, curve A. Potentiometric titration of mepivacaine hydrochloride in glacial acetic acid with 0.1 *N* perchloric acid in glacial acetic acid as a titrant and with mercuric acetate and crystal violet indicator added, curve B.

The common parenteral forms of mepivacaine hydrochloride used in dentistry are 1.8-ml. cartridges which contain 3% local anesthetic or 2% local anesthetic with a vasoconstrictor at a concentration of 1:20,000. For types of anesthesia other than dental, 1 and 2% mepivacaine hydrochloride solution are available in single and multiple-dose vials containing as much as 50 ml.

The ultraviolet absorption of local anesthetic solutions is frequently a convenient and accurate method of assay. While the ultraviolet absorption of mepivacaine hydrochloride is distinctive (Fig. 3), even the more intense band at 263 mμ with an $E(1\%, 1\text{ cm.})$ of 16.5 is somewhat low for assay purposes. The absorption spectrum of the base and the hydrochloride salt in methanol are essentially identical with that of the hydrochloric salt in

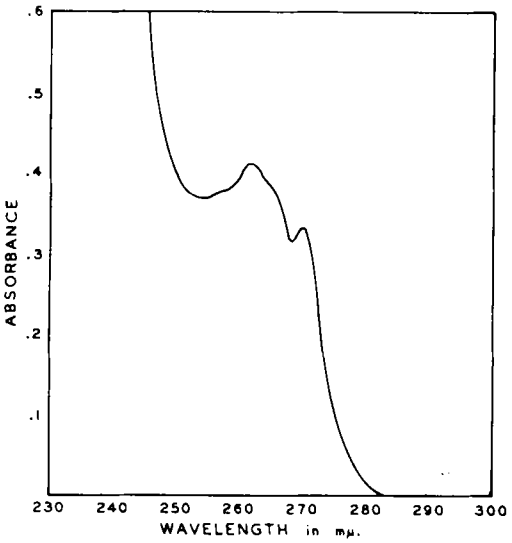


Fig. 3.—Ultraviolet absorption of mepivacaine hydrochloride in water.

The *cis*-aconitic anhydride method for tertiary amines (4) has been used successfully to determine mepivacaine hydrochloride in the usual aqueous solutions as well as blood and serum samples. Mepivacaine base has been used as a standard for this colorimetric procedure. This method may not be suitable for routine analysis, especially for the assay of the usual mepivacaine hydrochloride solutions where the advantages of this method are not fully utilized.

The mepivacaine hydrochloride in the usual local anesthetic solution can be determined gravimetrically as mepivacaine base by extraction with methylene chloride or chloroform of a solution of the hydrochloride which has been made alkaline with sodium carbonate. In such a procedure, the phenolic vasoconstrictors form water-soluble sodium salts which remain in the alkaline aqueous phase while the mepivacaine base is extracted into the organic phase.

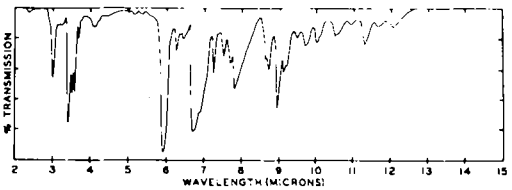


Fig. 4.—Infrared absorption of mepivacaine base in chloroform.

The mepivacaine base obtained from an extraction procedure can be determined by infrared absorption (Fig. 4). The absorption of mepivacaine base in chloroform at 5.97 μ in a 1.0-mm. sodium chloride cell obeys Beer's law in the range 0–6 mg./ml. The method involves the extraction of the base from an alkaline solution with chloroform. The chloroform extract is evaporated in a jet of warm air to less than 10 ml. and then diluted with chloroform to 10 ml. in a volumetric flask. The absorption at 5.97 μ of this solution in a 1.0-mm. cell *vs.* a chloroform-containing reference cell is compared with a standard. This standard, which is a sample of mepivacaine hydrochloride of known purity, is carried through the same procedures as the unknown. Using the baseline technique, the standard deviation of this method is 1.0%.

Mepivacaine base, subsequent to an extraction procedure, can be determined by metachromatic or potentiometric titration with 0.01 *N* perchloric acid in dioxane (Fig. 2). The direct titration of the base in the chloroform extraction solvent makes this a rapid, convenient method. The standard deviation of this titrimetric method is 1.0% and, unlike the ultraviolet and infrared methods, does not require a mepivacaine reference standard.

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Gas Chromatography of Barbiturates II. Application to the Study of Their Metabolism and Excretion in Humans

By A. BAERHEIM SVENDSEN and E. BROCHMANN-HANSSEN

Five different barbiturates were administered to adult humans and their urine analyzed for the unchanged and metabolized products by gas chromatography. Barbital was easily identified in urine collected 16 days after administration. The demethylation of N-methyl substituted barbiturates could be followed. *p*-Hydroxy phenobarbital was detected by gas chromatography after administration of phenobarbital and mephobarbital.

DEVELOPMENT of new and more specific methods of analysis has repeatedly led to reinvestigation of problems associated with the metabolism and excretion of barbiturates. The earliest studies were based on gravimetric methods which, for the most part, lacked specificity and sensitivity (1–7). More recently, ultraviolet spectrophotometry has been extensively used (7–23). Occasionally, erroneous results have been reported due to the fact that the spectrophotometric method usually does not differentiate between an unchanged barbiturate and its metabolic product. Paper chromatography (24–26) and liquid-liquid partition systems (20, 27) have been used for separation of such mixtures. Gas chromatography has been shown to be a rapid and selective method for analysis of barbiturates in complex mixtures (28–30). It seemed to be of interest to study the applicability of this method to biological systems, particularly with regard to the urinary excretion of certain barbiturates and their metabolites.

EXPERIMENTAL

The experiments were carried out with adult humans. A single, therapeutic dose of the barbiturate in powder or tablet form was taken in the evening, and the urine collected the next morning and at intervals during the following 24 to 40 hours. In the case of barbital, urine samples were also collected 6 and 16 days after administration.

Purification.—The total volume collected during each time interval was acidified with dilute hydrochloric acid and extracted several times with ether. The combined ether extracts were washed with a 1% solution of sodium bicarbonate which removed much of the colored impurities. The bicarbonate wash was shaken once with a little ether, which was then combined with the total ether extract. The barbiturates were extracted from the ether solution with 0.1 *N* sodium hydroxide. Each alkaline extract was filtered through a pledget of cotton into an excess of dilute hydrochloric acid. The combined and acidified aqueous extracts were shaken several times with ether, the ether extracts dried over anhydrous sodium sulfate and evaporated to dryness in a rotating vacuum evaporator. The residue was dissolved in 1 to 5 ml. acetone, and this solution was used for analysis.

Gas Chromatographic Analysis. The instrument and experimental conditions described previously were used (30). The quantitative determinations were based on planimetric measurements of peak areas and internal standardization. Most of the work was carried out on a column containing 1.7% of neopentyl glycol sebacate on acid- and alkali-washed Chromosorb W, 60–80 mesh. However, *p*-hydroxy phenobarbital could not be eluted from this column. Apiezon L was found to be a satisfactory stationary phase for separation of phenobarbital and its *p*-hydroxy derivative.

The results are given in Tables I to V.

DISCUSSION

Although the present work is based on only a single administration of each drug, the results are in good agreement with earlier reports. Of the five barbiturates investigated, only barbital appears to be excreted quantitatively in unchanged form. Thus, following a single dose of 1500 mg., Lous (15) reported a recovery from urine of 33% after 48 hours and of 75 to 95% after 10 to 14 days. Our own work also confirms this slow elimination of barbital. Even after 16 days, detectable amounts were present in the urine.

No metabolite has ever been isolated from aprobarbital. Although Fabre (4) has reported to have recovered 46 to 90% of the dose after 6 to 10 days, more recent work (15) shows recoveries of less than 20% after 5 to 8 days. In our experiment, 8% of the dose was found unchanged after 48 hours. These low recoveries of aprobarbital would lead one to suspect that a major portion of this barbiturate may be metabolized to substances that are not detected by the methods used so far.

Butler, *et al.* (21), have shown that dogs excrete phenobarbital mainly as the *p*-hydroxy derivative. Later Curry (24, 25), Algeri and McBay (26), and Butler (20) have found that this conversion also takes place in humans, although to a lesser extent. In dogs, *p*-hydroxy phenobarbital is almost completely conjugated, mainly with glucuronic acid, while humans excrete about one-half of the metabolite in the free form, the other half as a conjugate other than the glucuronide (20). *p*-Hydroxy phenobarbital interferes with the U.V. spectrophotometric determination of phenobarbital unless a separation is carried out prior to the determination (27). By gas chromatography on a polyester column, only phenobarbital was eluted and determined. However, a column containing Apiezon L as the stationary phase (1.4% on Chromosorb W, 60–80 mesh) gave a well-defined peak for the *p*-

Received November 7, 1961, from the University of California, School of Pharmacy, Medical Center, San Francisco 22.

Accepted for publication January 2, 1962.

This work was supported by a research grant (M-3487) from the National Institutes of Health.

TABLE I.—URINARY EXCRETION AFTER SINGLE ADMINISTRATION OF 300 MG. OF BARBITAL

Time After Administration	Urine Volume, ml.	Barbital Found— mg.	Barbital Found— Cum. % of Dose
0–8.5 hr.	300	6.6	2.2
8.5–17 hr.	250	12.1	6.2
17–24 hr.	300	14.7	11.1
24–32 hr.	300	15.9	16.4
6 days (8-hr. sample)	300	5.2	...
16 days (24-hr. sample)	900	Trace	...

TABLE II.—URINARY EXCRETION AFTER SINGLE ADMINISTRATION OF 200 MG. OF METHARBITAL

Time After Administration, hr.	Urine Volume, ml.	Found— Metharbital— mg.	Metharbital— Cum. % of Dose	Barbital— mg.	Barbital— Cum. % of Dose
0–9.5	500	0.54	0.27	0.26	0.13
9.5–24	550	0.66	0.6	3.18	1.72
24–48	900	1.17	1.18	15.1	9.37

TABLE III.—URINARY EXCRETION AFTER SINGLE ADMINISTRATION OF 200 MG. OF PHENOBARBITAL

Time After Administration, hr.	Urine Volume, ml.	Found— Phenobarbital— mg.	Phenobarbital— Cum. % of Dose	*p*-OH-phenobarbital
0–9	400	6.3	3.65	+
9–24	550	15.5	10.9	+
24–48	900	25.0	23.4	+

TABLE IV.—URINARY EXCRETION AFTER SINGLE ADMINISTRATION OF 150 MG. OF MEPHOBARBITAL

Time After Administration, hr.	Urine Volume, ml.	Found— Mephobarbital mg.	Mephobarbital Cum. % of Dose	Phenobarbital mg.	Phenobarbital Cum. % of Dose	*p*-OH-Phenobarbital
0–9	400	1.2	0.8	6.4	4.3	+
9–24	300	Trace	...	6.3	8.5	+
17–32	500	Trace	...	6.8	13.0	+

TABLE V.—URINARY EXCRETION AFTER SINGLE ADMINISTRATION OF 150 MG. OF APROBARBITAL

Time After Administration, hr.	Urine Volume, ml.	Aprobarbital Found mg.	Aprobarbital Found Cum. % of Dose
0–9.5	300	1.7	1.1
9.5–24	350	1.8	2.3
24–48	1,350	8.7	8.1

hydroxy derivative. At 187° the retention time was 5.5 minutes for phenobarbital and 20 minutes for the metabolite. Authentic samples of *p*-hydroxy phenobarbital isolated from dog urine[1] and synthesized according to Butler (20) were used to identify the metabolite in urine collected after administration of phenobarbital and mephobarbital. No attempts were made to determine the amounts of the *p*-hydroxy compound quantitatively. However, a visual inspection of the chromatograms showed the amounts of the unconjugated material to be smaller than those of phenobarbital.

[1] The authors are indebted to Dr. Thomas C. Butler for supplying us with a sample of this material.

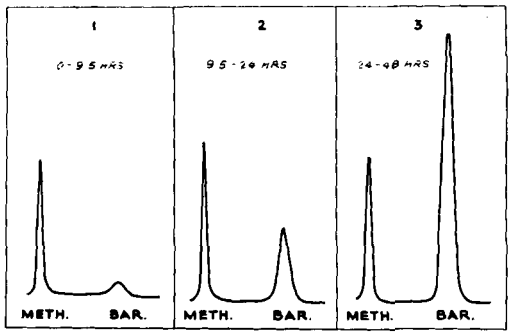


Fig. 1.—Gas chromatograms of urine extracts after administration of metharbital. METH., metharbital; BAR., barbital.

N-Methyl derivatives of barbiturates are demethylated in the body. Butler (16, 18, 22) has shown that mephobarbital is much more rapidly demethylated than metharbital. Our results confirm this (Tables II and IV, Fig. 1).

CONCLUSION

The gas chromatographic method of analysis has been found to be suitable for identification and quantitative determination of barbiturates and their metabolites excreted in the urine. Results obtained with five barbiturates are in good agreement with earlier reports.

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Study of the Color Reaction of Streptomycin Sulfate with Procaine Hydrochloride

By A. V. PUCCINI and A. J. SPIEGEL

The cause of the increased color of streptomycin sulfate solution in the presence of procaine hydrochloride was studied. It was determined that the carbonyl function of the streptomycin reacted with the *p*-amino group of the procaine causing a discoloration beyond that of a normal streptomycin solution.

THE PURPOSE of this study was to investigate the cause of an increased darkening of streptomycin sulfate solution in the presence of procaine hydrochloride and to determine the functional groups responsible for this reaction.

This was of interest since procaine hydrochloride is frequently added to streptomycin sulfate solutions used for intramuscular or subcutaneous injection to alleviate pain at the injection site.

The streptomycin molecule is composed of three parts: streptidine, streptose, and N-methyl-L-glucosamine, connected to one another by glycosidic linkages (1). The presence of a reactive carbonyl group can be shown by its ability to undergo typical aldehyde reactions such as the formation of the oximes and semicarbazones (2). The preparation of biologically active N'-alkylstreptomycylamines have also been reported (3). Dihydrostreptomycin, due to the absence of this carbonyl group, is, therefore, a more stable, less reactive compound.

Streptomycin solution is subject to irreversible destruction by acid or alkali, and the rate of decomposition increases as the pH is lowered below 3.0 or elevated above 8.0. This decomposition is due to the hydrolysis of streptomycin producing streptidine and streptobiosamine (4). Streptomycin solutions normally darken upon standing. The extent and rate of color change depend in part on the purity of the preparation; however, this change in color is not necessarily accompanied by loss of activity.

EXPERIMENTAL

The streptomycin sulfate and dihydrostreptomycin sulfate used in this study were U.S.P. grade. The solutions used contained 400 mg. of antibiotic activity per ml. of an aqueous citrate solution, buffered at pH 6.0. The samples were stored at 50° in a constant temperature oven for various periods of time to accelerate reaction conditions. The absorbance was read on a Bausch and Lomb Spectronic 20 colorimeter at 450 mμ. The increase in absorbance under these conditions was a measure of the rate of color formation.

Solutions of streptomycin sulfate with procaine hydrochloride, tetracaine hydrochloride, and lidocaine hydrochloride in 2% concentrations were studied to determine their effect on the color formation of streptomycin in solution. Controls of the aforementioned compounds were also investigated. Figure 1 indicates that tetracaine hydrochloride and lidocaine hydrochloride had no effect on the color formation of streptomycin solutions. Procaine hydrochloride, however, greatly increased the color intensity of these solutions. This indicated that the primary amino group in the procaine hydrochloride molecule was the functional group involved

Received October 30, 1961, from the Pharmaceutical Research Department, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.
Accepted for publication December 8, 1961.

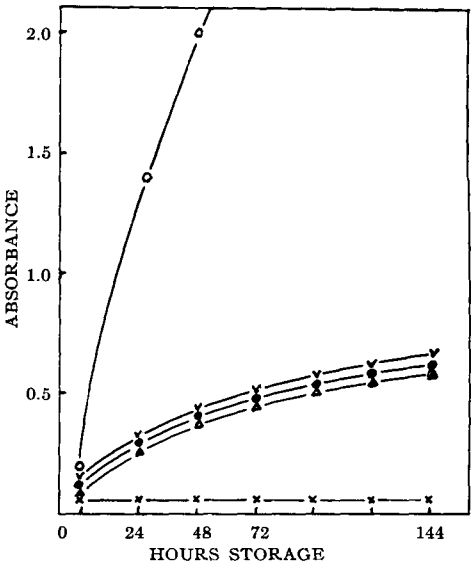


Fig. 1.—The effect of amines on streptomycin color formation. O, Streptomycin sulfate and 2% procaine hydrochloride; V, streptomycin sulfate and 2% tetracaine hydrochloride; ●, streptomycin sulfate; △, streptomycin sulfate and 2% lidocaine hydrochloride; x, 2% procaine hydrochloride, 2% tetracaine hydrochloride, and 2% lidocaine hydrochloride.

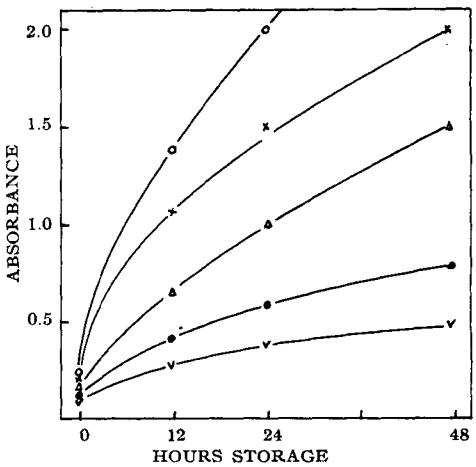


Fig. 2.—The effect of various concentrations of procaine hydrochloride on streptomycin color formation. V, Streptomycin solution; ●, streptomycin and 0.5% procaine hydrochloride; △, streptomycin and 1.0% procaine hydrochloride; x, streptomycin and 2.0% procaine hydrochloride; O, streptomycin and 4.0% procaine hydrochloride.

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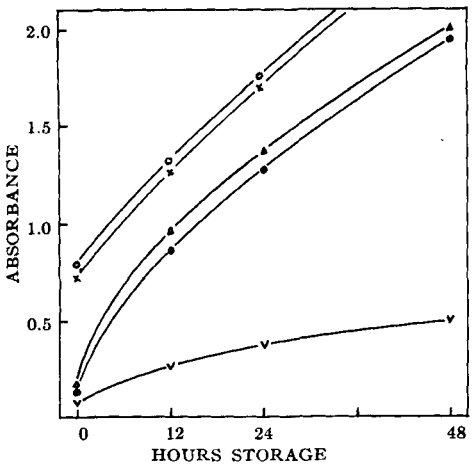


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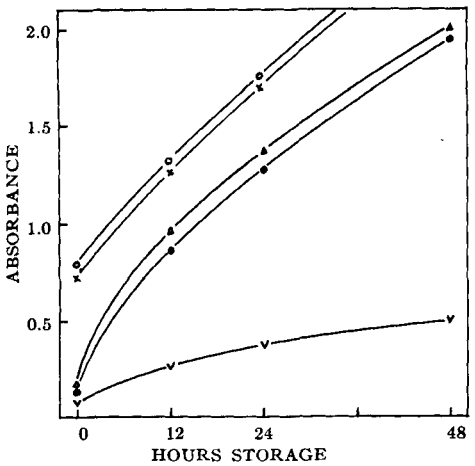


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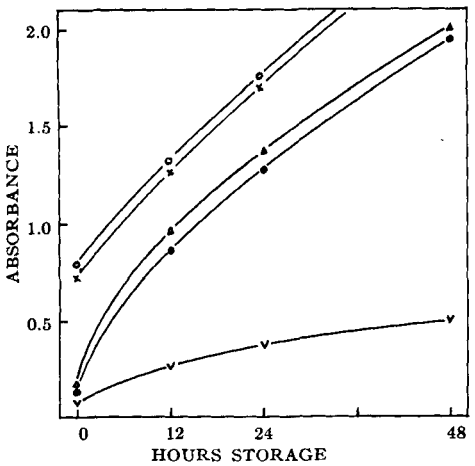


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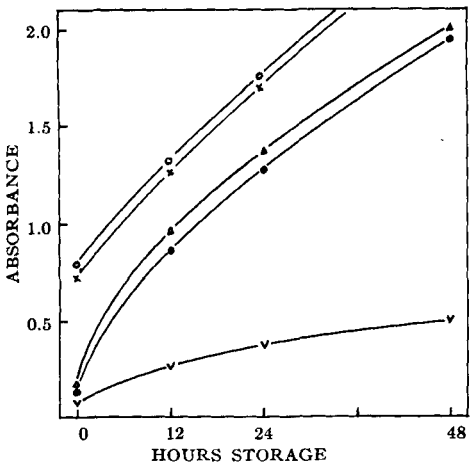


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The Givaudan Index. 2nd ed. Givaudan-Delawanna, Inc., 321 West 44th St., New York 36, N. Y., 1961. 431 pp. 15 × 23 cm. Price $7.50.

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Chromatography. Edited by ERICH HEFTMANN. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1961. xxv + 753 pp. 15 × 22.5 cm. Price $17.50.

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This book is designed for use as a textbook in biochemistry for the first year medical student. It contains much recent information that would interest anyone in this and related fields. New material relates to: absorption, transport, mobilization, and intermediary metabolism of lipids; structure and biosynthesis of proteins; structure and metabolism of nucleic acids; abnormal hemoglobins; biochemical genetics; metabolic antagonism; classification and mechanism of action of enzymes; enzyme kinetics; regulation of the volume and composition of body fluid compartments; calcium homeostasis and skeletal mineralization; metabolism of steroid and adrenal medullary hormones; neurohormones; metabolism in nervous and adipose tissue.

Textbook of Physiology and Biochemistry. 5th ed. By GEORGE H. BELL, J. NORMAN DAVIDSON, and HAROLD SCARBOROUGH. The Williams and Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1961. xi + 1,117 pp. 16.5 × 24 cm. Price $13.50.

A comprehensive British textbook for students in the health sciences. It is liberally provided with graphs and figures as visual aids to understanding and illustrating the text. A good index is appended.

Nervous Inhibition. Edited by ERNST FLOREY. Pergamon Press Ltd., Headington Hill Hall Oxford, England, 1961. xv + 475 pp. 15 × 23 cm. Price $15.

A compilation of 31 papers presented at the May 31, 1960 symposium held at the University of Washington, Friday Harbor Laboratories. General topics covered include the development of theories of inhibition, inhibition in the mammalian spinal cord, inhibition of receptor nerve cells, nervous inhibition of endocrine systems, and presynaptic inhibition. Author and subject indexes are appended.

Hypertension—Recent Advances. Edited by ALBERT N. BREST and JOHN H. MOYER. Lea & Febiger, 600 South Washington Square, Philadelphia 6, Pa., 1961. xxi + 660 pp. 15 × 23 cm.

The papers in this compilation were presented at the second Hahnemann Symposium on Hypertensive Disease in Philadelphia, May 1961. The seven main groups include related papers on: Natural history of hypertension, Etiological mechanisms, Atherosclerosis, Pharmacology, Catecholamine metabolism and Drugs affecting it, Therapeutic considerations (22 papers in latter group). A general index is appended.

Abriss der Laboratoriumstierkunde. Edited by G. HOFFMANN. Veb Gustav Fischer Verlag, Jena, Villengang 2, Germany, 1961. xviii + 270 pp. 16.5 × 24 cm. Price DM 39.50.

This book (German) describes the various laboratory animals and gives in detail their utilization in animal experimentation.

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JOURNAL OF

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF **Pharmaceutical Sciences**

June 1962 volume 51, number 6

Review Article

Crystallography. Part I

By JOHN A. BILES

"The impossible we do at once—miracles take a little longer." (1)

INTRODUCTION

WILLIAM H. WERKMEISTER, a philosopher, once compared the advances of the sciences with those of the humanities (2). He showed that advances in travel could be illustrated by plotting velocity of travel by man or machine with date of discovery. The resulting plot was roughly exponential. The advances in the humanities with time, he suggested, would represent a zig-zag line which does not show the long range upswing. Since the discovery of crystallography, advances with time would fit the exponential-type curve. With little or no imagination, one concludes that X-ray crystallographers in the near future will be performing routine studies in structural analysis without the use of chemical properties. With increasing effort they will help to predict or substantiate hypotheses in reaction mechanisms. They have already helped to solve the riddle of life; scientists recognize the worth of the crystallographer when discussing RNA and DNA. X-ray crystallographers, through their reports, will allow scientists to utilize imaginations with increasing tempo. It is little wonder then that Grenville-Wells reminded the readers of the optimistic advertisement quoted above (1).

Cannon recorded a discussion with Gamgee, a British biological chemist who visited Cannon's laboratories at Harvard University at the turn of the century (3). After Cannon apologized for the physical facilities, Gamgee responded, "I have never noticed that the nature of the cage determined the singing of the bird." Today, the pharmaceutical crystallographer has been termed a "rare bird" (4), but this bird is the first to admit that the cage is important. The refinement of methods and X-ray equipment and the development of computors have contributed to a most rapid advance.

Crystallography was first developed as a branch of mineralogy. It was concerned with the study of three-dimensional bodies and the laws which governed their growth, shape, and geometric character. Crystallography soon reached such proportions that it became a separate science. To review crystallography in total would require volumes. Faced with this dilemma, the reviewer took the easy way out by limiting the pages of words to some aspects of X-ray and optical crystallography. To review just two of the total number of different phases of crystallography and application to pharmaceutical research does not allow one to conclude that there are but two important phases—it just provides an opportunity for other experts to write review articles.

Received from the University of Southern California, Los Angeles 7.

The author wishes to express his appreciation for the assistance offered by J. W. Shell, T. Higuchi, W. I. Higuchi, and J. G. Wagner.

Editor's note: Other important aspects of crystallography, including optical crystallography and crystalline properties, will be discussed by Dr. Biles in Part II of this review which will appear in the July issue of THIS JOURNAL.

NOMENCLATURE

An ideal crystal is a regular polyhedral solid bounded by plane faces which represents an extended array of atoms arranged in a definite order in all directions. This body contains a unit cell or unit of structure, repetition of which, in three dimensions, produces the crystal. Each unit cell is the same size for the specific crystal and contains the same number of particles similarly arranged. Even though size and shape of such a crystal may vary, the angles between the faces will remain constant. At times many of the crystal faces may not be evident, but there still remains an orderly internal arrangement of the atoms. A measurement of the interfacial angles reveals a number of faces of the same type. Faces of the same type are referred to as a form. In some instances, one form is all that is needed to define the three-dimensional solid. In other instances and at times demanded, two or more forms are present. The crystals of a given substance may vary in size, relative development of given faces, and the number and kind of faces or forms present. Crystals showing such variations have different habits. Thus, some crystals are described as needles, others are tabular, equant, columnar, or lamellar. The habit acquired depends upon the solvent used, the temperature, concentration, impurities, and the rate of precipitation.

The crystal faces are identified using Miller indexes. These indexes consist of a series of whole numbers which have been derived from the parameters by their inversion and, if necessary, the subsequent clearing of fractions. The numbers are so given so that the three numbers refer to the *a*, *b*, and *c* crystallographic axes, respectively. The possible faces from various planes of molecules or atoms, as identified by the Miller indexes may be illustrated by either two- or three-dimensional figures. Imagine looking down the *c* axis of a crystal and at the *a-b* plane of the crystal. Some different crystal faces which parallel and do not intersect the *c* axis are identified by Miller indexes, as shown in Fig. 1.

Crystal faces intersect at definite angles to each other and the faces of the crystal parallel the internal planes of the crystal. Thus, one may compare Fig. 1 with the observation of the density of trees in the various rows of trees in a fruit orchard. The next time you are driving down the highway and pass a fruit orchard, try to visualize the different planes and guess the Miller indexes for each of the rows. Perhaps, though, it would be simpler to consider the

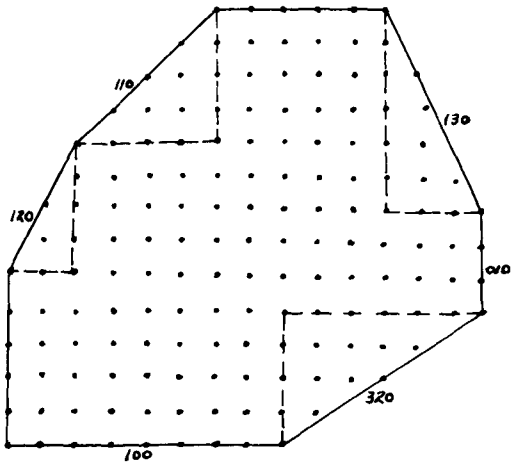


Fig. 1—A cross section of a crystal perpendicular to the *c* axis.

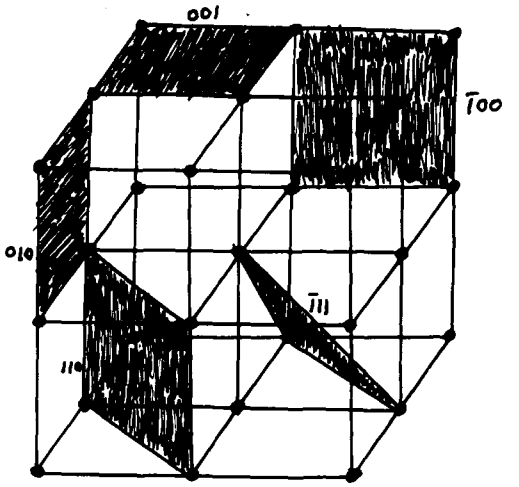


Fig. 2—Some possible crystal faces of the sodium chloride crystal.

structure of the sodium chloride crystal in which one sodium ion is surrounded by six chloride ions, and each chloride ion surrounded by six sodium ions. The possible faces could be identified by the three-dimensional diagram illustrated in Fig. 2.

Examining Fig. 2, it is observed that there is a perfectly regular arrangement of the cations and anions. The three-dimensional network of sodium ions represents the space lattice of the sodium ions in the crystal of sodium chloride. Similarly, there is a space lattice of chloride ions. The space lattice of sodium chloride represents the interpenetration of the sodium and chloride lattices. Each ion in the lattice has exactly the same environment as any other point representing the same ion. The space lattice of the crystal is built up of a three-dimensional basic pattern

which is called the unit cell. The external appearance of the crystal is determined by the shape and dimensions of the unit cell.

By the application of geometry, it has been shown that only 14 different kinds of simple space lattices are possible. That is, there are only 14 ways in which similar points can be arranged in a regular three-dimensional order. By taking combinations of the various lattices that are possible for each crystallographic system, there have been built up 230 different space groups. The space-group symbols and the procedure for deducing the space groups are described in crystallography textbooks (5). For purposes of review, most of the readers recall that crystals vary in their angular relationships and symmetry. On this basis, a crystal is classified into one of six crystal systems. Each system is defined in terms of the crystallographic axes, which are imaginary lines used to describe the position of the plane faces in space. The systems, depending on the symmetry, are subdivided into 32 classes.

X-RAY CRYSTALLOGRAPHY

The increasing importance of this science can be felt by reading the excellent reviews. One could read the fascinating reports of molecules in crystals (6), the motion of the atoms in crystals (6, 33), crystallographic studies of compounds of biological interest (34, 35), the application of crystallography to chemistry (36), the use of X-ray crystallography in the pharmaceutical industry (37, 38), and British achievements in X-ray crystallography (39). Should the scientist become a convert to this science, in a few sentences Lonsdale could tell him the required background (40). If a few hundred pages are necessary to convert the scientist, then out of the many excellent textbooks written, the books of Bunn (41) and Robertson (42) would prove interesting material.

The Unit Cell

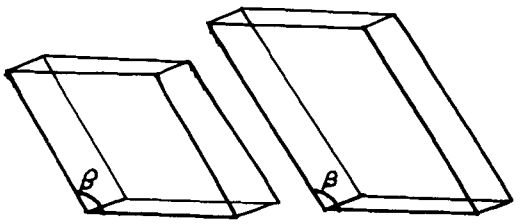
The arrangement of the molecules in the space lattice and the relationship of the molecules to the crystal shape was predicted before the use of X-ray procedures. Hodgkin (6) and Robertson (7) have called attention to the observations of Hooke in 1664. Hooke noted the crystal shape of alum. He considered that the shape resulted from "three or four several positions or postures of globular particles . . . by these kinds of texture or position of globular bodies, may you find out all the variety of regular shapes into which the smooth surfaces of Alum are form'd. . . nor does it hold in superficies but in solidity also, for it's obvious that a fourth Globule laid upon the third in this texture, composes a regular Tetrahedron, which is a very usual Figure of the Crystals of Alum."

By X-ray analysis, it has been determined that each unit cell contains four aluminum ions, four potassium ions, 48 water molecules, and eight sulfate ions. Hodgkin commented that, remarkably enough, the relative positions of the positive ions in the crystal conform with Hooke's suggestions but, in total, the structure of alum is considerably more complicated than Hooke's drawings propose (6).

One of the first insights into the unit cell structure was presented by W. H. Bragg when he measured the unit cells of naphthalene and anthracene (8). The measurements were also reported by Robertson in 1933 (9, 10). The cell dimensions for the two compounds are shown in Table I and Fig. 3.

TABLE I.—UNIT CELL DIMENSIONS OF SOME AROMATIC HYDROCARBONS

	a, Å.	*b*, Å.	*c*, Å.	*β*
Naphthalene	8.34	6.05	8.69	122°49′
Anthracene	8.58	6.02	11.18	125°
Diphenyl	8.22	5.69	9.5	94.8°
p-Diphenylbenzene	8.08	5.60	13.59	91.9°
p-Diphenyl-diphenyl	8.05	5.55	17.81	95.8°



(A) (B)

Fig. 3—The unit cell dimensions for naphthalene (A) and anthracene (B).

It should be noted that two sides of the unit cells in Fig. 3 are almost the same distance. Also the angle beta (obtuse angle) for both cells are very close. The two cells differ with respect to the *c* axis, which is 2.49 Å. longer in anthracene than in naphthalene. It may be recalled that the diameter of the benzene ring is almost this distance (8).

The unit cell dimensions have also been determined for diphenyl (11), *p*-diphenylbenzene (12), and *p*-diphenyldiphenyl (13). The unit cell dimensions for these compounds are also listed in Table I. Again two sides of the cells are very similar (also similar to those of naphthalene and

anthracene); the angle beta for the three compounds are similar, but the difference lies along *c*. The bond distance between the benzene rings was found to be 1.48 Å. Adding the 1.48 Å. to the 2.49 Å., the sum is found to be approximately the same value as the differences between the values along *c*.

The unit cell dimensions of long chain organic compounds have been the subject of discussion for approximately 30 years. Francis, Collins, and Piper (14) determined the unit cell dimensions for a number of saturated fatty acids, alcohols, alkyl iodides, and ethyl esters, as well as some dicarboxylic acids. A definite correlation was obtained when the molecular weight of the compounds of each of the homologous series was plotted against the melting point. A straight line relationship was also obtained when the crystal spacings were plotted against the carbon content. Francis, *et al.*, reported that when the fatty acids containing the even numbered carbon atoms were crystallized from glacial acetic acid, C-type crystals were obtained. The specific spacings were noted. When the same fatty acids were crystallized from either benzene or acetone, different spacings were noted. These were referred to as B crystals. When the fatty acids containing odd numbered carbon atoms were crystallized from glacial acetic acid, acetone, or benzene, only spacings corresponding to B crystals were observed. Abrahamsson and von Sydow (15) reported the variation of unit cell dimensions of long chain fatty acids of form C. For the acids with 12, 14, 18, 22, and 26 carbon atoms, it was found that all dimensions in the monoclinic cell were found to be dependent on the number of carbon atoms in the chain. The *a* and *b* axes and angle beta decreased asymptotically with increasing chain length. The *c* axis and long spacings, *d* (001), obeyed linear laws.

Warren and Matthews (16) noted the straight line relationship of *d* spacings and molecular weight for alcohol derivatives of the xanthates. They used the strong inner line as the *d* distance of choice. They also investigated the unit cell dimensions of the anilides of acids from formic acid through stearic acid (17). The patterns of the even members of series C_8 to C_{18} show a marked similarity, suggesting a uniformity of crystal structure which may be described as isostructural. Their results were in agreement with Slagle and Ott (18). Matthews, Warren, and Michell continued this line of investigation (19). When the first-order reflections of the silver salts of fatty acids were plotted against the number of carbon atoms in the fatty acid, a straight line was obtained. The equation was determined to be

$$X = 9.85 + 2.44\,(y - 2) \qquad \text{(Eq. 1)}$$

The value of 2.44 represented the increment of the —CH_2 group, and the value of 9.85 represented the combined value for the —CH_3 and —COOAg group. Similarly, in a study of the amides of the fatty acids, the straight line relationship of the first-order reflection was given by

$$X = 6.25 + 1.85\,(y - 2) \qquad \text{(Eq. 2)}$$

The value of 1.85 for the amides represented the increment for the —CH_2 group.

Merritt and co-workers identified alkyl halides as the alkyl 6-nitrobenzothiazolyl-2-sulfides and sulfones (20). Their differentiation of the halides was made by observation of the three strongest reflection lines. Wurz and Sharpless studied the amides of saturated aliphatic acids (21). A straight line relationship was correlated for the odd and even series. The line for the even series had a greater *y* intercept than that for the odd series. Garska, *et al.*, recently reported that when the fatty alcohol could not be identified with gas chromatography or mass spectrometry, the alcohol benzoate could be prepared and X-ray analysis performed (22). They too reported an apparent correlation existing between length of the carbon chain attached to the 3,5-dinitrobenzoate radical and the size of the largest *d* spacing in the diffraction pattern of the ester.

Sharma and Biswas reported the X-ray diffraction study of *n*-alkyl malonic acids (23). The melting points of the acids and the characteristic long spacings were recorded. Their work is quite similar to those reported by Francis, *et al.* (14). The saw tooth arrangement of the melting points and long spacings for the odd and even membered series was reported. The spacings in the alkyl fatty acids corresponding to the length of the molecules suggest that the molecules are interlinked as dimers. Sharma and Biswas reported that such spacings in the alkyl malonic acids were approximately equal to the calculated extended length of a single molecule. They suggested that the alkyl malonic acids existed as a single unit, presumably due to internal saturation through intramolecular hydrogen bonding. Additional reports on relationships between the unit cell dimensions and chain length include those of Brock and Hannum (24), Lutz and co-workers (25), Guertin, *et al.* (26), and W. S. Singleton (27).

Pharmaceutical scientists could very well apply the knowledge obtained from unit cell dimensions. Such data can be used for purposes of

identification. The crystallographer may compare his diffraction pattern with the values in the A.S.T.M. file (28). In one of the laboratories it was reported that one of the liquid multivitamin preparations produced had a wine base as a major constituent. Upon changing the source of supply for the wine, a precipitate began to appear in the finished product. X-ray patterns were taken of the precipitate and checked against an A.S.T.M. file. The precipitate was identified as calcium tartrate tetrahydrate (29). Purity determinations can be determined. Concentration and stability has been suggested for suspensions (30). Shell states, since the crystalline material has a definite size unit cell, that this fact can be used as a general quantitative method of analysis which offers a combination of specificity and precision. The completeness or progress of a chemical reaction could be followed by X-ray diffraction methods since the unit cell dimensions of the product would, in all probability, be different from those of the reactants (31).

Mott has discussed the role of vacant lattice sites in crystalline solids. He believes that the vacant lattice site allows the diffusion in solids, and particularly when two metals are welded together. During the welding, a region intermediate between the two metals is formed which is occupied by an alloy of the two. Diffusion has taken place, which means that two adjacent atoms in a crystalline solid are able to change places if the temperature is high enough. This exchange can come about only with the help of a vacant lattice site. It was also expressed that vacancies play a role in metallic fatigue (32).

X-Ray Diffraction

In 1949, Kaufman and Fankuchen, reviewing the reports on X-ray diffraction, stated that in a matter of 10 to 15 years diffraction had been established as a most useful tool for analytical purposes. The tool was not self-sufficient; it was incapable by itself of replacing chemical analysis; but it had proved useful for identification of crystalline substances (43). Later reviews were written by Fankuchen, Kaufman, and Post (44–47). Post and Fankuchen also reported on low temperature X-ray crystallography (48). In 1936 there was concern about the filing of diffraction data. Hanawalt and Rinn suggested an effective use of a large library of standard X-ray diffraction patterns for the identification of unknowns (49). In 1938 Hanawalt, *et al.*, published information concerning classification and use of diffraction patterns. They included data on 1000 compounds (50). Frevel, Rinn, and Anderson then tabulated the diffraction data for 705 cubic substances (51), 447 tetragonal substances (52), and 1287 hexagonal substances (53). Matthews (54) recommended the use of a punched card system, and Ashley and Newton (55) suggested the use of a Kardex system. The American Society of Testing Materials (A.S.T.M.) card index file of X-ray diffraction data is widely used (56). Recently, Beukelman discussed the efficient use of an I.B.M. file of A.S.T.M. powder X-ray diffraction data (57). The literature sources for the X-ray diffraction patterns for different series of compounds of pharmaceutical interest are listed in Table II.

TABLE II.—REFERENCES TO X-RAY DIFFRACTION PATTERNS OF COMPOUNDS OF POSSIBLE PHARMACEUTICAL INTEREST

Compound Type	Compound Type
Aromatic hydrocarbons (58)	Azoic coupling components (77)
Aromatic hydrocarbons-trinitrofluorenone (59)	Cobalt ammine azides (78)
Alkyl halides (20)	Guanidine derivatives (79)
Aldehydes and ketones (60)	Explosives (80)
Surface-active agents (61)	Penicillins (81)
Fatty acids (27, 62)	Dyestuffs (82)
Fatty acid silver salts (26)	Barbiturates (83–85)
Aliphatic acid amines (21)	Sedatives and anticonvulsants (86)
Alkyl dithiol esters of sebacic acid (25)	Boron compounds (87)
Alcohol benzoate esters (22)	Phosphorous nitrogen compounds (88)
Substituted benzoic acids (63)	Sodium phosphates (89)
Aliphatic and aromatic amines (64)	Calcium phosphates (90)
Sterols and their digitonides (65, 66)	Strontium phosphates (91)
Steroids (67–70)	Crystalline ferrocenes (92)
Fructose dinitrophenylhydrazone (71, 72)	Copper chelates (93)
Phenols (73, 74)	Porphyrins (94)
Tetrazole derivatives (75)	Alcohols (95)
Oxadiazoles (76)	Bacterial viruses (117)

Some comments concerning the reports listed in Table II are warranted. Hofer and Peebles (58) reported that impurities lowering the melting point of the hydrocarbons as much as 15° did not modify the diffraction patterns appreciably. Rose and Van Camp (63) showed that benzoic acid derivatives having the same empirical formulas and same melting points could be differentiated with X-ray diffraction. Gaebler, Parsons, and Beher (65) detected differences in the X-ray diffraction analysis of digitonin polymorphs, whereas the infrared spectra of the samples used were virtually identical below 9.2 and above 9.7 μ. In the second study, Beher and co-workers reported that X-ray diffraction definitely established sterol digitonides as chemical com-

pounds, whereas infrared analysis did not distinguish between a simple mixture and a corresponding digitonide. Baker, Beher, and Parsons (67–70) reported the diffraction patterns for 138 steroids. Moore and Burkardt (75) stated that failure of X-ray patterns of the same compound to correlate may arise from polymorphism or preferential orientation of sample particles. In the reporting of the *d* distances for the various barbiturates, Williams (83) criticized the values reported by Penprase and Biles (84). Williams was justified in so doing if the *d* distances are required for pure solids, or for inclusion in the A.S.T.M. file. However, the report of Penprase dealt with a rapid identification of barbiturates using specific methods. With such methods polymorphism is evident and is, in fact, an aid for rapid identification. North and Rich (117) examined the T2 virus which has a very substantial tail structure and the T7 virus which has a small tail. Experiments suggested that some of the DNA inside the bacteriophage T2 virus head is oriented parallel to the tail. Furthermore, evidence suggested that the DNA may be organized into crystalline domains about 140 Å. across in T2 and 120 Å. in T7. The reports of the diffraction patterns of the various phosphates are indicative of the complexity of phosphate chemistry (89–91). The diffraction patterns of the calcium phosphates and strontium phosphates can undoubtedly be utilized in the study of strontium deposition in the bone. The review of the X-ray work of the fatty acids by O'Connor should be considered a masterpiece considering the complexity of the subject (62).

Quantitative Diffraction Method

Space will not permit the discussion of the use of X-ray diffraction analysis in a number of different analytical techniques. These techniques include emission spectroscopy (96–102), absorption (103, 104, 108), analysis combining absorption and diffraction (105), photoelectron spectra for surface analysis (106), low angle scattering analysis of particle size (107), and scattering methods (109,110). The selected references listed above are offered as a substitute for a discussion.

Single Crystal Analysis

Kaufman and Fankuchen (43) stated that single crystal diagrams may give more information than powder diagrams. In addition to a set of *d* spacings, one can obtain the unit cell size rather simply and, from a consideration of the systematic extinction, determine the space group. Bunn (41) has stated that the unit cell dimensions can be determined from powder diffraction data for the crystals of high symmetry. However, it is quite difficult to determine the unit cells from powder data for crystals of low symmetry. Nevertheless, Azaroff and Buerger have made available directions for the determination of unit cell dimensions from powder diagrams (111). This text is written in a manner which is easily understood. There are limitations in determining the unit cell dimensions. These limitations depend on the amount of data collected, particularly for a series of compounds. Examination of Table I will indicate the limitations and the value obtained from the unit cell determinations. Structural analysis is both much more complex and much more revealing.

Single crystal analysis is of great value in the determination of molecular weight. Douglass and Cook (112) reported that there was a question whether the lycoctonine monohydrate was represented by the empirical formula of $C_{25}H_{40}NO_7 \cdot H_2O$ or twice this value. The molecular weight determination by single crystal analysis was used to clarify the mystery. Straumanis (113) reported that the molecular weight determination by X-ray analysis may surpass the accuracy values obtained by chemical methods. The molecular weight of beta-lactoglobulin was determined by X-ray (114). Crystallographers in the pharmaceutical industry have been called upon to determine molecular weights by X-ray single crystal analysis (115, 116). Shell has described a method which requires a relatively small investment of time, yet with errors usually no greater than one hydrogen atom (116).

Structural Analysis

With continued refinement of methods used in the study of structural analysis, compounds have been restudied. It has become common in the past few years to calculate the electron density in crystals in three dimensions. With this study, the positions and modes of vibration of the atoms in the crystal may be studied (6). Cox, *et al.*, in a series of papers, studied the crystal structure of benzene (118–120). The positions of the hydrogens were determined and it was reported that the benzene molecule, as a whole, was oscillating with a root mean square amplitude of approximately 7.9° about the sixfold axis. Such a movement is allowed by the nondirected character of the intermolecular forces and the cog-wheel packing of the molecules in the crystal (6). The crystal structures of 3,4-benzpyrene (121) and 9,10-dimethyl-1,2-benzanthracene (122) were studied. Both molecules were found not to be planar. It was found that two ab-

normally short bonds and one very long bond were present in 3,4-benzpyrene. In 9,10-dimethyl-1,2-benzanthracene, ring D was found to be bent down out of the general plane by about 20° and atom C_{15} up by about 12°. On the other hand, in a study of the crystal structure of 1,2-benzanthracene, the molecule was found to be planar within experimental error (123). Perhaps a relationship can be established from X-ray analysis concerning carcinogenic activity of these type compounds.

Aromatic hydrocarbon complexes have been studied (59, 124–127). Aromatic nitro compounds have been found to complex with hydrocarbons. The stability of the complex has been ascribed to "polarization bonding" (125) between components. The complexation of 1,3,7,9-tetramethyl uric acid, T.M.U.A., and pyrene has been studied by De Santis and co-workers. The T.M.U.A. solubilizes polycyclic aromatic hydrocarbons in water to form 1:1 and 1:2 crystalline complexes from organic solvents. It was found that the T.M.U.A. molecules in pure form in the crystal state stack up with alternate molecules similarly arranged. Neighboring molecules are not mirrored but rotated 180° with respect to each other. When a 1:1 complex of T.M.U.A. and pyrene crystallized out, the molecules were found to be stacked in alternating fashion. Every other molecule in a stack was a T.M.U.A. molecule. The orientation of these molecules was similar. A 1:2 complex has been isolated. The author would anticipate that the arrangement in the crystal will be a 1:2:1:2 arrangement. Of course it would be interesting to see if a 1:3 complex and a 2:3 complex could be isolated.

De Santis (127), *et al.*, suggested, that in the T.M.U.A. crystal the second molecule paired was rotated from the other molecule by 180° since the $\overset{+}{N}—\overset{-}{O}$ bonds (dipoles) are roughly perpendicular to the long axis of the molecules and that interaction between dipoles is the main factor responsible for pairing. Thus dipole-dipole interaction may predominate over the hydrogen bonds. When a methyl or a keto group is attached to the nitrogen, the dipole bond is weakened. The report of Sutor (128, 129) may be related to this discussion. It was found by Sutor that caffeine and theophylline molecules pair up in the same plane. The paired molecules are illustrated in Fig. 4. The arrows identify the dipoles.

Since the methyl group on the nitrogen reduces the strength of the dipole, and since the unit cell of caffeine is slightly greater in volume than that of theophylline, the greater solubility in water

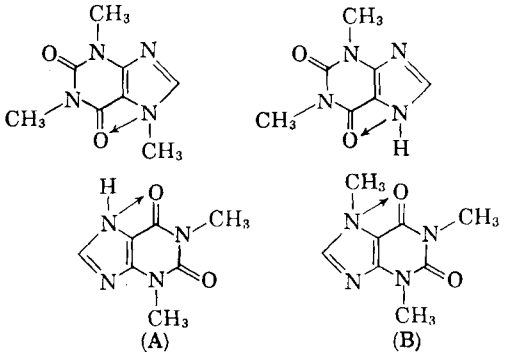


Fig. 4—The paired molecules of theophylline (A) and caffeine (B).

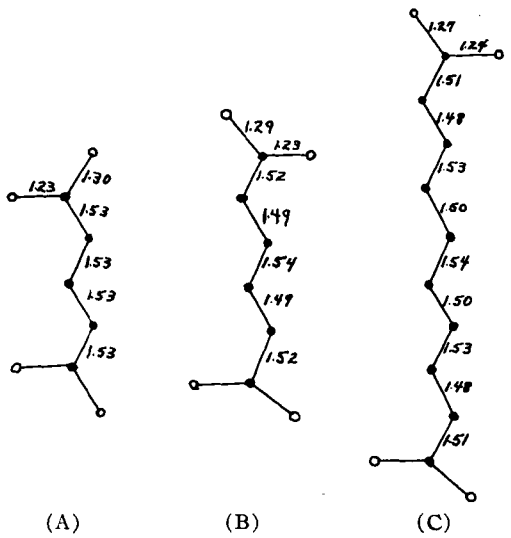
and lower melting point of caffeine, in comparison to theophylline, is better appreciated. Sutor points out that the disordering of the molecules along the *c* (of the unit cell) is the difference in the strength in which water is held. Theophylline does not effloresce even though there are chains of water molecules running through the structure. If the water molecules are disordered in their arrangement along *c*, only a fraction of them would be hydrogen bonded to the caffeine molecule. Thus, water is more firmly linked to a theophylline molecule. Gerdil and Marsh suggested an alternate proposal for the placement of water in the caffeine structure (130). With the information proposed concerning the relative positions of the molecules of caffeine and theophylline and the effect of the dipoles, the reader might want to make a comparison with the study of caffeine complexation by the Higuchi group (131–135).

The crystal structures of uracil (136), adenine hydrochloride (137), guanine hydrochloride (139), and thymine monohydrate (138) have been reported. Parry draws attention to the close similarity between the dimensions of the pyrimidine ring in adenine hydrochloride and in uracil. Broomhead reported that the cell dimensions of guanine hydrochloride and adenine hydrochloride are different, but the structures are strikingly similar. The structures of various nucleotides have been reported (140–142). Bryan and Tomita (143) emphasized the value of the study of purines and pyrimidines for use in the formulation of structures for nucleic acids. For this formulation the identification of sites of protonation of the bases at acid pH, knowledge of the molecular geometry of the various bases, and a possible model for the geometry of interbase hydrogen bonding in polynucleotides would be most valuable. The reading of Spencer's report on the stereochemistry of DNA should be a must for

those interested in cell development (144). The Crick and Watson model has suggested a fascinating mechanism for the process of heredity (39). The structure analysis of proteins has been reviewed by Kendrew and Perutz (35). A most recent review on proteins by Kendrew was published (145).

The difference in solubility in water and melting points for the aliphatic dibasic acids has been the subject of reports by X-ray crystallographers (146–150). Morrison and Robertson (150) stated that several factors could account for the saw tooth type arrangement. The carboxyl groups in beta-glutaric acid deviate further from the plane of the carbon atoms than in the even numbered series studied. The end-to-end hydrogen bridge connections between the carboxyl groups are 2.69 Å., which is almost the same as in the even structures, but the lateral connection is 3.6 Å., which is much weaker than in the even series. In addition, the bond lengths are more closely normal in beta-glutaric acid than those found in the even series. The bond distances of some of the dibasic acids studied (147–150) are shown in Fig. 5.

Bragg, Perutz, *et al.* (151–153), examined the structure of hemoglobin. They reported that the pleochroism of sheep hemoglobin varied considerably. On this basis they suggested that structural differences are evident between fetal and adult sheep hemoglobin (152). In a later paper they reported that reduced, oxy, carboxy, or methemoglobin existed in three polymorphic forms, whereas the hemoglobin of normal adult man crystallizes in six different forms. Patterson projections of horse and human hemoglobin possess similarities which imply a close relationship between the internal architecture of these two proteins (153). Partial X-ray crystallographic studies have been reported for the sterols (154) and three trimethylsteroids (155). The structural analysis has not been performed for these complex molecules. The unit cell dimensions have been published. This is also true of prednisolone acetate (155). Gafner, *et al.*, reported that the trimethyl steroids had many similarities. He cautioned, however, that the intensities of the diffraction patterns could be misleading since powder samples were not free from preferred orientations (155).



(A) (B) (C)

Fig. 5—The bond distances for glutaric acid (A), adipic acid (B), and sebacic acid (C).

A major accomplishment in X-ray structural analysis was performed by the Hodgkin group in England with the help of White, Prosen, and Trueblood in the United States (157–160). They determined the structure of vitamin B_{12} without the use of chemical analysis. The various techniques used in determining the structure would prove interesting reading.

X-ray structural analysis has been used to aid in the determination of molecule stability and in explaining organic reactions. Three examples will be discussed (161–166). The structure of benzene and *p*-chlorobenzene iododichloride has been studied (161, 162). Archer and van Schalkwyk reported that the ICl_2 group in benzene iododichloride was linear and symmetrical. The direction of this group is approximately at right angles to the *ac* plane. Bekoe and Hulme (162) stated that the electrophilic group in the *para* position gave rise to a dipole moment directed away from the iodine atom. It is possible that a tendency of electrons to drift away from the iodine atom partially increased the positive charge on the iodine and so makes a greater resonance stabilization of the molecule. In the linear ICl_2 group the third valence is at right angles to the halogen group. Presumably dsp^3 or d^3sp bonds are formed, but not fully used. The main cohesive forces in the crystal are between the iododichloride groups. Watson, in studying the crystal stuctures of 1-chloro-2,4-dinitrobenzene and 1-bromo-2,4-dinitrobenzene stated that the relative positions of the nitro group should be studied since this group affected the rate of reaction for nucleophilic aromatic substitution (163). A maximum activation through electron transfer is attained if the nitro group is planar with the benzene ring. It was reported that the nitro group in the 1-chloro derivative was rotated 39° and the nitro group was rotated 40° for the 1-bromo derivative.

Thus, the activating effect of the nitro group is greatly diminished by this steric effect and may result in a net deactivation. Donohue and co-workers have examined the polymorphs of sulfur (164–166). Donohue (164) suggested that the geometrical considerations of some of the allotropic forms of sulfur should be included in discussions concerning reactions of compounds containing the S—S bond, especially with regard to transition states or activated complexes, even though the precise energetics are not known. Thus the structural data of sulfur will undoubtedly be of increasing importance.

Recently, developments in techniques have allowed a reinvestigation of bond distances. Refinement of the structure of anthraquinone was reported by Murty (167). Refinement was done utilizing two-dimensional synthesis, three-dimensional synthesis, and three-dimensional differential synthesis. The hydrogen atoms were detected and their positions verified. Thus the hydrogen carbon bond distance was measured and compared to that obtained by other methods. The structure of chlortetracycline hydrochloride was also analyzed by X-ray procedures (168). From the analysis of this antibiotic Hirokawa, *et al.*, proposed that (*a*) the fourth ring takes a partial quinone structure, (*b*) C_{11} and O_{11} are single bonded, (*c*) C_{10a}—C_{12} is conjugated and no double bond is localized on the C_{11a}—C_{12} bond, (*d*) carbon sequence C_1—C_3 is also conjugated, (*e*) carbon sequence C_4—C_6 is completely saturated, (*f*) the dimethylamino group takes the polar configuration with respect to the first ring. Point (*f*) represents an exception to the structure found on chemical grounds. Thus the X-ray structure corresponds to the conformation assigned on chemical grounds to epi-chlortetracycline. The organic portions of the molecules exhibit only van der Waals contacts except for the hydrogen bonds around the anion. Only intramolecular hydrogen bonds are found. The chloride ion, which lies near the apex of the pyramid made by the dimethylamine group and C_4 is surrounded by six atoms which form a distorted octahedron. Those six atoms are N_4, O_6, $CH_{3(6)}$, O_{12}, O_{12a}, and N_{amide}.

Crystallographic studies of the biuret reactions have been made by Freeman and co-workers (169). They report that the metal-peptid complexes appear to be involved *inter alia* in the transition states of metal-activated proteolytic enzyme reactions. The metal contributes to the color formation. In some studies for metal binding the peptid nitrogen atoms were the donor atoms while in other studies the donor atoms were the peptid oxygens. When copper II was chelated and involved the nitrogen atom, the complex was anionic divalent; when chelation occurred with the oxygen, the complex was cationic divalent. The crystal structure of isosteric compounds was studied. Giacomello and co-workers determined the orientations and stucture of 2,4-dihydroxybenzoic acid and 2-hydroxy-4-aminobenzoic acid. It was found that even though the compounds were isosteric, crystallization occurred and different lattices were formed. The relationship of the isosteres in the crystalline state was illustrated. The reader should refer to the original reference for full appreciation (170, 171). X-ray studies of additional compounds of pharmaceutical interest are listed in Table III.

TABLE III.—SOME COMPOUNDS OF PHARMACEUTICAL INTEREST ANALYZED BY X-RAY

Acridine polymorphs (172–174)	Chelates (179)	Penicillin (185,186)
dl-Alphaprodine HCl (175)	Colchicine (180)	Strychnine (187,188)
Antihistamines (176)	Cycloseri ne(181)	Liquid water (189)
Biotin (177)	Fatty acids (62, 191)	Ice (190)
Cellobiose (178)	Formic acid (182)	
	Glycine polymorphs (183, 184)	

The X-ray analysis of colchicine (180) was helpful in the determination of the structure of the molecule. von Sydow (191) illustrates the various forms of the fatty acids referred to as A, A′, B, B′, C. Malenkow proposed that in liquid water the importance of the dodecahedron formation from water molecules is evident.

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Research Articles

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The absorption of tolbutamide and several of its salts was studied by means of carboxytolbutamide excretion rate measurements following the oral ingestion of the drugs in nearly constant surface dosage forms by normal adult humans. Lowering of blood sugar levels produced in normal adult humans after the oral ingestion of the same preparations was also studied. Initial rate of decline of blood sugar level could be correlated with either the absorption rate of tolbutamide or the amount of tolbutamide in the body 1 hour after drug ingestion; increasing with increasing absorption rate or increase in amount of tolbutamide in the body. In most instances, increases in *in vitro* dissolution rate were reflected in increases in rate of decline of blood sugar level, absorption rates, or amounts of tolbutamide in the body at 1 hour.

THE RELATIONSHIP between the rate of absorption of the antidiabetic drug tolbutamide[1] and resulting depression in blood sugar level has not beeen previously studied. The availability of several salts of tolbutamide with different dissolution rates and, hence, different absorption rates, made possible the present study which was concerned with the nature of this relationship.

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Tolbutamide is 1-butyl-3-*p*-tolylsulfonylurea.

EXPERIMENTAL

Materials.—Pharmaceutical grade tolbutamide and its sodium salt were used.

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Tolbutamide is 1-butyl-3-*p*-tolylsulfonylurea.

EXPERIMENTAL

Materials.—Pharmaceutical grade tolbutamide and its sodium salt were used.

The 2-amino-2-methyl-1-propanol salt of tolbuta-

mide was prepared in the following manner: 100 Gm. (0.37 mole) of tolbutamide was added to about 1200 ml. of deionized water, previously heated to 50°. To this slurry was added, in small portions, 34.8 Gm. (0.37 mole) of 2-amino-2-methyl-1-propanol (tech. grade). Stirring and heating were continued and a small additional amount of the amine (1.0 Gm.) was added to effect complete solution of the acid. The hot solution was filtered, placed in a lyophilizing flask, and frozen.

Following the removal of the solvent by lyophilization, the resulting light, fluffy, white solid was triturated with successive portions of ether in a porcelain mortar. The resulting slurries were filtered and the filter cake was washed with fresh portions of ether. The solid was dried for 48 hours *in vacuo;* yield, 131 Gm., m.p. 150.2–153.8°.

Anal.—Calcd. for $C_{16}H_{29}N_3O_4S$: C, 53.46; H, 8.13; N, 11.69; S, 8.92; equiv. wt., 359. Found: C, 53.77; H, 8.36; N, 11.52; S, 8.99; equiv. wt., 353; % H_2O (Karl Fischer), none.

The 1-amino-2-propanol salt of tolbutamide was prepared by the following procedure: to 1200 ml. of deionized water heated to 50° was added 100 Gm. (0.37 mole) of tolbutamide. To the resultant slurry 28 Gm. (0.37 mole) of 1-amino-2-propanol (tech. grade) was added in small portions with constant agitation. An additional amount (1 Gm.) of the amine was added to effect a complete solution. The hot solution was filtered, placed in a lyophilization bottle, and frozen.

The white, fluffy solid produced upon removal of the aqueous phase by lyophilization was triturated with small portions of ether in a porcelain mortar. Following filtration of the resulting slurries, the filter cake was washed with fresh portions of ether. The solid was dried *in vacuo* for 68 hours; yield, 90 Gm., m.p. 91.4–94.6°.

Anal.—Calcd. for $C_{15}H_{27}N_3O_4S$: C, 52.15; H, 7.88; N, 12.16; S, 9.28; equiv. wt., 345. Found: C, 52.17; H, 7.91; N, 11.75; S, 9.37; equiv. wt., 345; % H_2O (Karl Fischer), none.

Dosage Forms.—The sodium, 2-amino-2-methyl-1-propanol, and 1-amino-2-propanol salts of tolbutamide, as well as tolbutamide free acid, were compressed into thin cylindrical disks at a pressure of about 40,000 p.s.i., using commercial tableting equipment. The disks were 1.28 cm. in diameter and their thickness varied from 0.168 cm. to 0.229 cm., depending on the form of tolbutamide used. Each disk contained 250 ± 10 mg. tolbutamide equivalent and had a surface area of 3.38 ± 0.09 cm.[2].

Test Subjects and Conduction of Tests.—Two separate test panels were used: one for the studies on the absorption and excretion rates of carboxytolbutamide[2] following the ingestion of 0.5 Gm. tolbutamide in the various forms, and the other for studying blood sugar level following ingestion of 1.0-Gm. doses of tolbutamide in the same forms. The test subjects for all experiments were normal adult humans. Drug was ingested on fasting stomachs in the morning and no food was taken until 1 hour had lapsed from the time of drug ingestion in the absorption experiments. In the blood sugar lowering tests the compounds were administered after overnight fasting and no food was taken during the active course of the experiments (10 hours).[3]

Analytical Methods.—Urine was assayed for carboxytolbutamide by a previously described method (1). Blood sugar determinations were made on blood obtained from the antecubital veins by means of an Autotechnicon. The analytical method was that of Hoffman (2).

In Vitro Dissolution Rate Studies.—Dissolution rates of each of the forms were determined in 0.1 *N* hydrochloric acid and 0.2 *M* tris(hydroxymethyl)-aminomethane (adjusted to pH 7.2 with hydrochloric acid) solutions at 37° using a method previously described to study dissolution rates of slightly soluble weak acids and their sodium salts (3).

RESULTS

Excretion Experiments.—Cumulative excretion of carboxytolbutamide following the ingestion of tolbutamide in the several hours is given in Table I.

Blood Sugar Level Studies.—Results of blood sugar tests in 23 normal subjects are shown in Tables II and III.

In Vitro Dissolution Rate Studies.—The dissolution rates *in vitro* determined by the method described are summarized in Table IV.

DISCUSSION

Treatment of Excretion Data.—Cumulative amounts of carboxytolbutamide excreted in 1, 2, 3, and 4 hours by each of the test subjects in each test were fitted to polynomials with powers of time of the form

$$Y = at + bt^2 + ct^3 + dt^4 \qquad \text{(Eq. 1)}$$

where Y was the amount excreted in mg. and t was time in hours. The coefficients of Eq. 1, for each of the tests, are listed in Table V.

It has been previously reported that absorption rate (A.R.) may be calculated from excretion measurements by the following expression (4)

$$\text{A.R.} = \frac{1}{f}\left(\frac{dAe}{dt} + \frac{1}{K}\frac{d^2Ae}{dt^2}\right) \qquad \text{(Eq. 2)}$$

In Eq. 2, dAe/dt is excretion rate, d^2Ae/dt^2 its derivative, K the rate constant for elimination of drug, and f the fraction of drug reaching circulation that is excreted unchanged. All quantities in Eq. 2 refer to unchanged drug. Tolbutamide is nearly quantitatively oxidized to carboxytolbutamide *in vivo* and this metabolite is quantitatively excreted in the urine about 12 times more rapidly than it is formed (5). Therefore, even though carboxytolbutamide excretion was followed, this very closely represented tolbutamide's oxidation and application of Eq. 2 to calculate absorption rate should not result in the introduction of any substantial error. In order to calculate absorption rate of tolbutamide by Eq. 2 the first and second derivatives of Eq. 1 were taken at 1.0, 1.5, and 2.0 hours. A mean value of K for each of the subjects used in the excretion experiments was available from other work (5), hence absorption rate could be calculated at the

[2] Carboxytolbutamide is *p*-[(butylcarbamoyl)sulfamoyl]-benzoic acid.

[3] The experimental design used in the blood sugar lowering tests is shown in the Appendix.

TABLE I.—CUMULATIVE MG. CARBOXYTOLBUTAMIDE EXCRETED FOLLOWING INGESTION OF TOLBUTAMIDE IN SEVERAL FORMS

Time, hr.	Subject E	Subject I	Subject B	Subject C	Time, hr.	Subject E	Subject I	Subject B	Subject C
	1-Amino-2-propanol Salt					Sodium Salt			
1	25	5	23	21	1	28	9	18	27
2	82	34	80	65	2	73	40	67	78
3	127	73	138	112	3	122	81	122	123
4	173	113	187	190	4	163	119	166	164
6	241	176	270	278	6	236	174	243	220
8	298	232	320	338	8	278	227	307	277
10	343	281	362	387	10	328	273	340	316
12	374	320	395	432	12	364	312	370	345
24	468	467	534	547	24	437	433	490	444
48	541	535	586	567	48	514	502	559	490
Theory[a]	555	555	555	555	Theory[a]	537	545	545	555
	2-Amino-2-methyl-1-propanol Salt					Free Acid			
1	7	4	12	2	1	6	3	11	1
2	34	14	30	9	2	10	5	12	2
3	68	29	57	36	3	19	8	16	4
4	107	49	93	75	4	30	13	19	9
6	190	91	173	163	6	47	25	26	21
8	243	129	235	232	8	61	37	46	33
10	289	167	281	292	10	71	49	66	46
12	334	203	326	344	12	84	59	98	54
24	434	375	481	502	24	124	105	180	93
48	489	480	565	602	48	219	182	216	124
Theory[a]	547	555	555	555	Theory[a]	569	569	569	569

[a] 1.11 × dose of tolbutamide taken.

TABLE II.—AVERAGE DIFFERENCES IN BLOOD SUGAR, IN MG. %, IN 0 TO 1-HOUR AND 0 TO 2-HOUR PERIODS FOLLOWING ORAL ADMINISTRATION OF THE EQUIVALENT OF 1 GM. OF TOLBUTAMIDE IN THE FORM OF DISKS[a]

Form of Tolbutamide	Difference in Blood Sugar Level, mg. %: 0–1 hr. Av.	0–1 hr. S.E.	0–1 hr. 95% Confidence Interval of Av.	0–2 hr. Av.	0–2 hr. S.E.	0–2 hr. 95% Confidence Interval of Av.
1-Amino-2-propanol salt	27.2	1.8	24.0–31.4	17.0	1.0	14.9–19.1
Sodium salt	19.1	2.3	14.3–23.9	23.7	1.8	20.0–27.4
2-Amino-2-methyl-1-propanol salt	16.0	1.5	12.9–19.1	20.0	1.2	17.5–22.5
Tolbutamide (acid)	5.2	1.3	2.5– 7.9	7.0	1.1	4.7– 9.3

[a] 23 Normal subjects.

TABLE III.—ADJUSTED[a] AVERAGE BLOOD SUGARS IN MG. % AND AS PER CENT OF PLACEBO GROUP AVERAGE AT EACH HOUR[b]

Form of Tolbutamide	Hours After Administration[c]: 0.5	1	2	4	6	8	10
1-Amino-2-propanol salt	...	53.0	63.4	67.3	72.4	73.7	67.3
	...	68.6	83.2	92.8	96.1	99.6	99.0
Sodium salt	62.4	57.6	68.3	70.9	70.6	70.3	70.0
	76.4	73.9	84.7	90.2	93.6	93.2	97.3
2-Amino-2-methyl-1-propanol salt	...	70.1	66.2	69.2	70.0	68.8	66.5
	...	83.2	82.2	85.6	88.8	91.5	91.5
Tolbutamide (acid)	...	82.0	79.8	78.7	77.1	74.2	72.1
	...	97.7	97.4	97.0	97.8	99.7	101.7

[a] Adjusted for difference in initial blood sugar level by covariance analysis. The zero-hour blood sugar level was used as a covariate. [b] 23 Normal subjects for disks and 5 normal subjects for placebo. [c] Top row, average blood sugars in mg. %; bottom row, per cent of placebo group average.

indicated times for each of the preparations. The calculated rates are listed in Table VI in terms of tolbutamide equivalent (1 Gm. tolbutamide = 1.11 Gm. carboxytolbutamide). The value of K for subjects E, I, B, and C in Tables I, V, VI, and VII were, respectively, 0.139, 0.105, 0.133, and 0.124 hours^{-1}.

When the mean values of absorption rate, following ingestion of the sodium or 1-amino-2-propanol salts of tolbutamide were plotted *vs.* time on semilogarithmic graph paper, the line was linear indicating that absorption was by an apparent first-order process. This is shown in Fig. 1. With the 2-amino-2-methyl-1-propanol salt, the absorption

rate was nearly constant in the 2-hour period where the calculations were made. The differences in the nature of absorption processes was not unexpected. Both the sodium and 1-amino-2-propanol salts of tolbutamide dissolve rapidly in mediums possessing hydrogen ion concentrations in the physiological range (Table IV). The 2-amino-2-methyl-1-propanol salt of tolbutamide and tolbutamide free acid dissolve substantially slower and, since the dosage forms could be expected to have presented a nearly constant surface to dissolution fluids *in vivo* due to their geometry, this dissolution process was apparently rate limiting in absorption in the time period under consideration.

The excretion rate of carboxytolbutamide is very nearly directly proportional to the amount of tolbutamide in the body (5). That is,

$$dTe/dt \cong KT \qquad \text{(Eq. 3)}$$

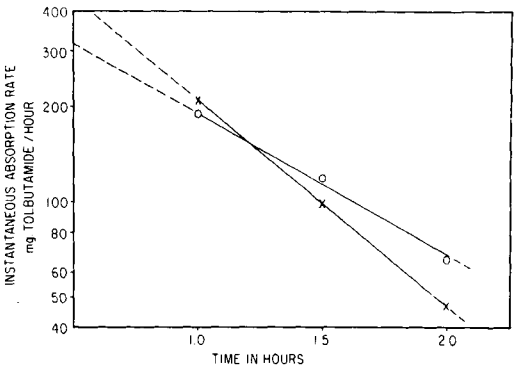


Fig. 1.—First-order plots of absorption rate. ×, 1-Amino-2-propanol salt of tolbutamide; ○, sodium salt of tolbutamide.

Table IV.—*In Vitro* Dissolution Rate of Tolbutamide in Several Forms

Form of Tolbutamide	Rate, mg./cm.²/hr. 0.1 *N* HCl	Rate, mg./cm.²/hr. 0.2 *M* Tris-(hydroxymethyl)-aminomethane[a]
1-Amino-2-propanol salt	264 (207)[b]	358 (280)[b]
Sodium salt	1156 (1069)	938 (868)
2-Amino-2-methyl-1-propanol salt	0.37 (0.28)	19 (14)
Tolbutamide (acid)	0.21 (0.21)	3.1 (3.1)

[a] Adjusted to pH 7.2 with hydrochloric acid. [b] Rate expressed in terms of tolbutamide equivalent.

where dTe/dt is the excretion rate of carboxytolbutamide, K the rate constant for the *in vivo* oxidation of tolbutamide in hours^{-1}, and T is the amount of tolbutamide in the body at any time. The value of T for each of the subjects in each of the tests was calculated at 1 hour and these are listed in Table VII in terms of tolbutamide equivalent. This was done by using the first derivative of Eq. 1.

Correlation Between Absorption Rate or Body Level of Tolbutamide and Initial Lowering of Blood Sugar Level.—The rate of lowering of blood sugar level was examined in respect to two parameters concerned with tolbutamide absorption. One of these was the rate of initial depression of blood sugar level compared to the half-life for absorption. This

Table V.—Coefficients of the Polynomial
$Y = at + bt^2 + ct^3 + dt^4$

Subject	a	b	c	d	Subject	a	b	c	d
	1-Amino-2-propanol Salt of Tolbutamide					Sodium Salt of Tolbutamide			
E	−19.9	64.1	−21.6	2.38	E	14.9	15.4	−2.42	0.024
I	−12.9	21.3	−3.58	0.021	I	−5.75	16.5	−1.75	−0.042
B	−10.7	44.0	−11.2	0.096	B	−7.83	31.7	−6.17	0.33
C	−9.17	43.5	−15.3	2.00	C	−3.00	41.7	−13.0	1.33
	2-Amino-2-methyl-1-propanol Salt of Tolbutamide					Tolbutamide (Acid)			
E	−10.08	21.5	−4.92	0.458	E	11.8	−9.08	3.67	−0.42
I	0.417	3.96	−0.417	0.042	I	4.42	−1.96	0.58	−0.42
B	10.7	0.125	1.25	−0.125	B	24.6	−19.3	6.42	−0.71
C	10.2	−15.5	8.25	−0.958	C	1.08	−0.04	−0.08	0.04

Table VI.—Absorption Rate of Tolbutamide at 1, 1.5, and 2 Hours after Ingestion of Several Forms[a]

Subject	1.0 hr.	1.5 hr.	2.0 hr.	Subject	1.0 hr.	1.5 hr.	2.0 hr.
	1-Amino-2-propanol Salt of Tolbutamide				Sodium Salt of Tolbutamide		
E	216	48	0	E	139	104	67
I	211	156	110	I	202	161	116
B	250	133	48	B	231	153	83
C	167	59	31	C	191	62	0
Mean	211	99	47	Mean	191	120	66
	2-Amino-2-methyl-1-propanol Salt of Tolbutamide				Tolbutamide (Acid)		
E	137	93	63	E	0	25	42
I	55	52	52	I	0	3	9
B	53	69	79	B	0	1	21
C	49	127	81	C	0	3	7
Mean	74	85	69	Mean		8	20

[a] Rate expressed in mg./hr. tolbutamide equivalent.

TABLE VII.—EXCRETION RATE IN MG./HR. TOLBUTAMIDE EQUIVALENT AND AMOUNT OF TOLBUTAMIDE IN MG. IN THE BODY 1 HOUR AFTER ORAL INGESTION OF 500 MG. TOLBUTAMIDE IN SEVERAL FORMS

Subject	Rate	Amount	Subject	Rate	Amount
1-Amino-2-propanol Salt of Tolbutamide			Sodium Salt of Tolbutamide		
E	46.1	333	E	33.7	244
I	17.1	163	I	19.1	181
B	41.2	309	B	33.3	250
C	24.6	280	C	40.7	328
Mean		271	Mean		251
2-Amino-2-methyl-1-propanol Salt of Tolbutamide			Tolbutamide (Acid)		
E	17.5	126	E	2.6	19
I	6.3	59	I	1.8	17
B	12.3	92	B	2.1	16
C	0.1	1	C	0.8	6
Mean		70	Mean		14

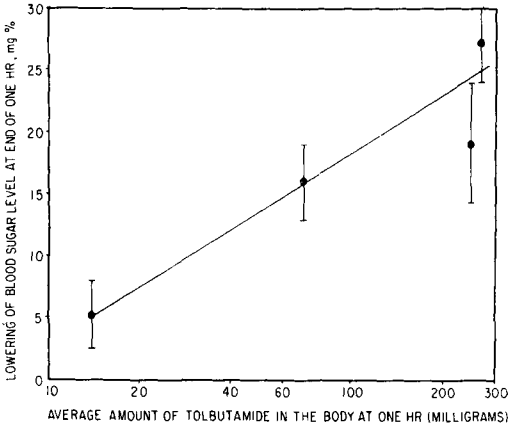


Fig. 2.—A plot of the lowering of blood sugar level at end of 1 hour against the average amount of tolbutamide in the body at 1 hour (on a logarithmic scale). Vertical bars mark off 95% confidence intervals of average blood lowering values.

comparison was only applicable in the case of the sodium and the 1-amino-2-propanol salts of tolbutamide since it was only with these materials that an apparent first-order absorption resulted (Fig. 1). From Table II the average rate of lowering of blood sugar level during the first hour was 27.2 and 19.1 mg. %/hr. for the 1-amino-2-propanol and sodium salt of tolbutamide, respectively. From Fig. 1, the half-lives for absorption were 0.46 and 0.68 hours, respectively; indicating the dependency of blood sugar level lowering on absorption rate.

In order to include all four test preparations in a correlation, lowering of blood sugar level at the end of the first hour of experiments is compared to the logarithm of the calculated amount of tolbutamide in the body at the same time on Fig. 2. The tendency for linearity indicates that a diminishing rate in blood sugar lowering is obtained with higher levels of tolbutamide. Examination of data on extent of initial lowering of blood sugar level as a function of dose of tolbutamide published by West and Johnson (6) indicates that the same general relationship between extent of lowering and dose holds.

Results obtained with the disks of tolbutamide (free acid) in these studies should not be taken as indicative of results which would be obtained with commercial tablets. In these studies the surface area of the tolbutamide was restricted to a very low

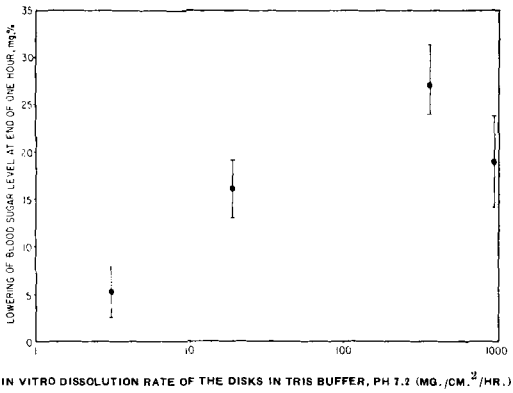


Fig. 3.—A scatter plot of lowering of blood sugar level at end of 1 hour in human subjects and *in vitro* dissolution rate of the tolbutamide dosage form in tris buffer, pH 7.2 (logarithmic scale).

TABLE VIII.—SCHEDULE OF TREATMENTS ASSIGNED TO POOL OF SUBJECTS AT RANDOM IN THE BLOOD SUGAR LOWERING TESTS

Subject No.	DAY 1	DAY 5	DAY 8	DAY 12
1	T[a]	O	T	O
2	O[b]	T	T	T
3	T	T	T	T
4	T	T	T	T
5	T	T	T	T
6	T	P	T	P
7	O	T	T	T
8	P[c]	T	T	T
9	T	O	P	P
10	T	T	O	T
11	T	O	P	T
12	T	T	T	T
13	T	T	O	T
14	T	T	T	T
15	O	P	T	O
16	T	T	T	T
17	T	O	T	P
18	T	P	T	T
19	T	..	..	..
20	T	P	O	P
21	T	T	T	T
22	T	T	P	T
23	T	T	T	O
24	O	T	P	P
25	P	T	T	T
26	T	T	T	T
27	P	P	..	..
28	P	O	T	T
29	T	T	O	T
30	T	T	O	O
31	P	T	T	T
32	O	T	T	T
33	T	T	T	T
34	..	T	T	O
35	..	..	P	T
Totals	23 T 5 P 5 O	23 T 5 P 5 O	23 T 5 P 5 O	23 T 5 P 5 O

[a] On day 1, T = free acid; on day 5, T = 2-amino-2-methyl-1-propanol salt; on day 8, T = sodium salt; on day 12, T = 1-amino-2-propanol salt. [b] O, a compressed tablet of tolbutamide. [c] P, a placebo.

value. The commercial tablets[4] disintegrate in the gastrointestinal tract liberating drug in fine particle size, and the results (7) are distinctly different.

Correlation Between Initial Lowering of Blood Sugar Level or Amount of Tolbutamide in Circulation and *In Vitro* Dissolution Rate of the Preparations.—The biological response obtained in these experiments is related to the physical chemical property of dissolution rate of the preparations. This may be seen from an examination of Fig. 3 where extent of blood sugar lowering after 1 hour is compared to the *in vitro* dissolution rates of the disks that served as dosage forms in the experiments. The *in vitro* dissolution rates used in the plot were from determinations in the nearly neutral mediums (Table IV). A generally similar relationship holds if the rates in acidic mediums are used in the plot. Comparison of data in Table IV to corresponding data in Tables I, II, and III also indicates the dependency of extent of carboxytolbutamide excretion or blood sugar level lowering, respectively, on dissolution rate.

SUMMARY

A study was made of the influence of the rate of absorption of tolbutamide on the initial depression of blood sugar levels in normal adult humans. Initial depression in blood sugar level is related to the absorption rate (increasing with increasing rate of absorption), amount of tolbutamide in the body in the time taken for comparison (increased with increased amounts of tolbutamide in the body), and to the *in vitro* dissolution rate of the various salts of tolbutamide used in the tests. Ultimately, differences in either depression of blood sugar level or amount of tolbutamide in the body during the times at which the comparisons were made was dependent on the *in vitro* dissolution rate of the preparations which surely determined *in vivo* absorption rate in this *in vivo*, solution rate-limited absorption.

[4] Orinase tablets, The Upjohn Co.

APPENDIX

Experimental Design Used in the Blood Sugar Lowering Tests.—A pool of 35 normal human subjects were used. Subjects were assigned at random to one of three treatments, namely; T, one of the test preparations in the form of cylindrical disks; O, a compressed tablet of tolbutamide; P, a placebo tablet. Nine subjects (39% of the 23 receiving the test preparations) were administered all four of the test preparations. The schedule of treatments is shown in Table VIII.

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Separation of Amino Acids on Ion Exchange Resin Papers

Dependence of R_f Values on pH and Ionic Strength

By A. BAERHEIM SVENDSEN and E. BROCHMANN-HANSSEN

Fifteen amino acids have been chromatographed on paper impregnated with an ion exchange resin of the sulfonic acid type. The R_f values have been determined at various pH and ionic strengths of the buffer eluant. By means of several chromatograms of appropriate pH values and ionic strengths, most amino acids can be separated and identified in about 2 hours. The ion exchange resin papers can be regenerated and used again.

DURING our studies of the amino acid composition of opium (1–3), various methods of separation and quantitative determination of amino acids have been investigated. Two-dimensional paper chromatography is satisfactory for most qualitative work, whereas the ion exchange procedure of Moore and Stein (4–6) is often preferred for quantitative analysis because of its accuracy and reproducibility.

In recent years papers impregnated with ion exchange resins have become available. These combine many of the advantages of paper chroma-

Received April 10, 1961, from the University of California Medical Center, School of Pharmacy, San Francisco.
Accepted for publication July 6, 1961.
This work was supported by a research grant (M-3487) from the National Institutes of Health, Bethesda, Md.

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In recent years papers impregnated with ion exchange resins have become available. These combine many of the advantages of paper chroma-

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tography with those of the ion exchange method, and several investigators have reported on the application of such papers to the separation of amino acids. Lautsch, Manecke, and Broser (7), as well as Tuckerman, Osteryoung, and Nachod (8), separated certain amino acids on carboxylated ion exchange paper. Myhre and Smith (9), using an aqueous-organic solvent, obtained results that were quite different from those which one would expect on the basis of ion exchange phenomena alone. Partition effects apparently modified the migration rates. Tuckerman (10) separated arginine, histidine, and lysine in casein hydrolysates by means of paper impregnated with a sulfonated ion exchange resin. This method was later used for quantitative determinations by Roberts and Kolor (11).

In the column chromatographic method, all the major amino acids are separated by increasing the pH, the ionic strength, and the temperature during the elution. This paper reports the effect of pH and ionic strength on the separation of amino acids on ion exchange paper.

EXPERIMENTAL

Materials.—Amberlite SA-2 ion exchange paper was used. This is prepared from alphacellulose pulp and finely ground ion exchange resin of the sulfonic acid type (Amberlite IR-120). Citrate buffer solutions were prepared in the pH range of 2.2 to 6.5 with sodium ion concentrations ranging from 0.1 to 0.8 *N*. The buffer solutions described by Moore, Spackman, and Stein (6) were used as starting points and adjustments made as needed to include a wider range of pH and ionic strength.

Procedure.—Sheets of ion exchange papers were cut into sections, 22 inches long and 9 inches wide. The lower edge was cut in a saw-tooth fashion so as to facilitate the flow of liquid off the paper. The papers were placed in a chromatographic cabinet and washed overnight with a buffer solution of the same pH and ionic strength to be used for elution. The buffer was then replaced with water to rinse out the excess of salts, and finally the papers were suspended in air until they were almost dry. Five microliters of 0.02 *M* solutions of the various amino acids were applied as small round spots four inches from the upper edge of the papers and about one inch apart. The papers were again placed in the chromatographic cabinet and developed with appropriate buffer solutions. The descending method was used for this as well as for the previous activation and washing. After about 2 hours, when the solvent front had progressed about 15 inches beyond the point of sample application, the papers were removed from the cabinet, air-dried, and sprayed with a 0.5% solution of ninhydrin in ethanol. The papers were left for 1 hour at room temperature and then heated at 100° for 10 minutes. The R_f values were calculated on the basis of six to eight chromatograms for each amino acid. At pH values where the neutral and acidic amino acids travelled closely behind the liquid front, the spots tended to become diffuse and poorly defined.

The ion exchange papers could be used again several times. The amino acid spots and excess of ninhydrin were removed with alcoholic hydrochloric acid (about 1 *N* hydrochloric acid in ethanol). The papers were washed with water and treated with buffer as described above. Care must be exercised in handling the wet papers as they tear very easily.

RESULTS AND DISCUSSION

The R_f values obtained for 15 amino acids at various pH values and ionic strengths are given in Table I. The effect of pH is also illustrated in Fig. 1, while Figs. 2 to 5 show the effect of the ionic strength. It is apparent that both factors, pH and ionic strength, have a pronounced effect on the rate of migration of the amino acids. No complete separation of all amino acids can be achieved in one single run. However, by running several chromatograms of appropriate pH and ionic strengths, most amino acids can be separated and identified in a relatively short time.

For a preliminary, exploratory examination of an amino acid mixture of unknown composition, a buffer solution of relatively low pH and concentration will yield much information. At a pH of 3.5 and a sodium concentration of 0.2 *N*, a reasonably good separation of the majority of common amino acids may be accomplished (*cf.* Fig. 1). If several acidic and neutral amino acids are present, these are best separated at a still lower pH value, for example 2.2, and at sodium concentrations of 0.6 and 0.8 *N* (Fig. 2). Basic amino acids are easily separated in the pH range of 5 to 6 (Figs. 1 and 5).

The R_f value of γ-aminobutyric acid is very much affected by pH between 4.5 and 6.5, while phenylalanine and tyrosine show greater response to pH

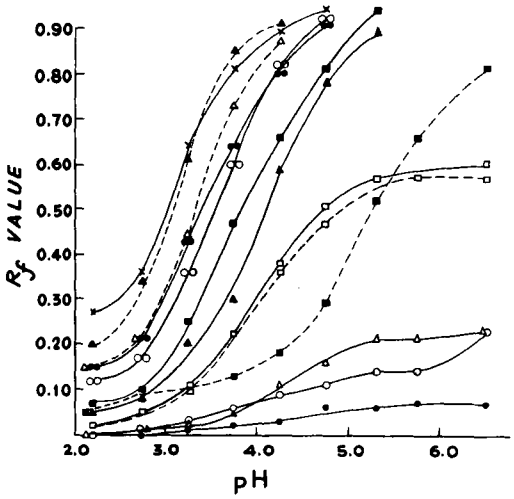


Fig. 1.—Influence of pH on the R_f values of arginine (—●—●—), histidine (—○—○—), lysine (—△—△—), tyrosine (--□--□--), phenylalanine (—□—□—), leucine (—▲—▲—), isoleucine (—■—■—), γ-aminobutyric acid (--■--■--), valine (—○○—○○—), alanine (—●●—●●—), glutamic acid (--△--△--), aspartic acid (--▲--▲--), and threonine (—×—×—).

TABLE I.—R_f VALUES OF AMINO ACIDS AT VARIOUS pH AND IONIC STRENGTHS

pH	2.20				2.75		3.25			3.75		4.25			4.75		5.28			5.75	6.50
[Na^+]	0.2	0.4	0.6	0.8	0.2	0.4	0.1	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8	0.2	0.4	0.1	0.2	0.4	0.6	0.8	0.2	0.2
Arginine	0	0.01	0.02	0.03	0	0.02	0.01	0.01	0.03	0.07	0.11	0.03	0.08	0.15	0.21	0.00	0.11	0.04	0.06	0.11	0.21	0.24	0.07	0.07
Histidine	0	0.02	0.05	0.10	0.01	0.08	0.01	0.03	0.10	0.21	0.26	0.06	0.19	0.30	0.41	0.11	0.22	0.07	0.14	0.23	0.39	0.45	0.14	0.23
Lysine	0	0.02	0.04	0.07	0.01	0.06	0.01	0.02	0.08	0.16	0.21	0.05	0.17	0.11	0.23	0.36	0.50	0.16	0.30	0.12	0.21	0.30	0.52	0.59	0.21	0.23
Tyrosine	0.02	0.04	0.07	0.10	0.05	0.10	0.04	0.10	0.15	0.26	0.26	0.22	0.26	0.36	0.41	0.47	0.55	0.47	0.51	0.54	0.57	0.58	0.60	0.60	0.57	0.57
Phenylalanine	0.02	0.04	0.07	0.07	0.05	0.12	0.06	0.11	0.17	0.26	0.24	0.22	0.27	0.38	0.41	0.45	0.52	0.51	0.52	0.56	0.57	0.58	0.60	0.57	...	0.60
γ-Aminobutyric acid	0.05	0.15	0.24	0.24	0.10	0.20	0.05	0.10	0.21	0.27	0.29	0.13	0.23	0.18	0.28	0.38	0.47	0.29	0.41	0.40	0.52	0.65	0.78	0.78	0.66	0.81
Leucine	0.05	0.10	0.15	0.15	0.08	0.19	0.10	0.20	0.29	0.43	0.43	0.30	0.38	0.59	0.67	0.74	0.77	0.78	0.84	0.80	0.89	...	...	...	...	..
Isoleucine	0.07	0.13	0.19	0.22	0.10	0.20	0.17	0.25	0.34	0.52	0.53	0.47	0.55	0.66	...	...	...	0.81	0.85	0.85	0.94	...	...	...	...	..
Valine	0.12	0.20	0.27	0.33	0.17	0.30	0.19	0.36	0.49	0.63	0.65	0.60	0.72	0.82	...	...	...	0.92	...	...	...	...	...	...	...	0.94
Alanine	0.15	0.25	0.34	0.40	0.21	0.32	0.22	0.43	0.54	0.71	0.72	0.64	0.75	0.80	...	...	...	0.91	...	...	...	...	...	...	...	..
Glycine	0.17	0.26	0.36	0.43	0.21	0.36	0.23	0.42	0.57	0.78	0.72	0.66	0.75	0.83	...	...	...	0.92	...	...	...	...	...	...	...	..
Glutamic acid	0.15	0.24	0.33	0.33	0.21	0.38	0.23	0.44	0.61	0.83	0.81	0.73	0.77	0.87	...	...	...	...	...	...	...	...	...	...	...	..
Aspartic acid	0.20	0.31	0.43	0.49	0.34	0.49	0.31	0.61	0.76	...	...	0.85	...	0.91	...	...	...	...	...	...	...	...	...	...	...	..
Serine	0.28	0.40	0.51	0.55	0.34	0.48	...	0.60	0.74	0.79	0.82	0.81	...	0.93	...	...	...	...	...	...	...	...	...	...	...	..
Threonine	0.27	0.41	0.53	0.63	0.36	0.48	...	0.64	0.75	...	0.84	0.81	...	0.89	...	...	...	0.94	...	...	...	...	...	...	...	..

Fig. 2.—Influence of ionic strength on the R_f values of arginine (—●—●—), lysine (—△—△—), histidine (—○—○—), tyrosine (--□--□--), phenylalanine (—□—□—), leucine (—▲—▲—), isoleucine (—■—■—), γ-aminobutyric acid (--■--■--), valine (○○—○○—○○), alanine (—●●—●●—), glycine (--○--○--), glutamic acid (--△--△--), aspartic acid (--▲--▲--), serine (--○○--○○--), and threonine (×—×—×).

Fig. 3.—Influence of ionic strength on the R_f values of arginine (—●—●—), lysine (—△—△—), histidine (—○—○—), tyrosine (--□--□--), phenylalanine (--□—□—), leucine (—▲—▲—), isoleucine (—■—■—), γ-aminobutyric acid (--■--■--), valine (○○—○○—○○), alanine (—●●—●●—), glycine (--○--○--), glutamic acid (--△--△--), aspartic acid (--▲--▲--), serine (--○○--○○--), and threonine (×—×—×).

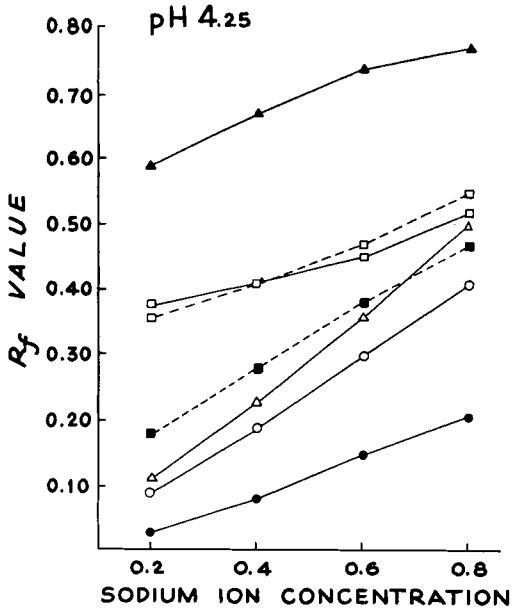


Fig. 4.—Influence of ionic strength of the R_f values of arginine (—●—●—), histidine (—○—○—), lysine (—△—△—), γ-aminobutyric acid (-·-■-·-■-·-) tyrosine (-·-□-·-□-·-), phenylalanine (—□—□—), and leucine (—▲—▲—).

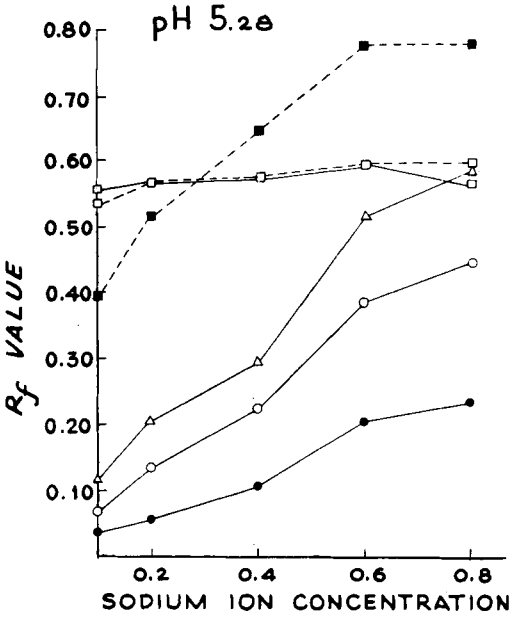


Fig. 5.—Influence of ionic strength of the R_f values of arginine (—●—●—), histidine (—○—○—), lysine (—△—△—), γ-aminobutyric acid (--■--■--), tyrosine (--□--□--), and phenylalanine (—□—□—)

changes at lower pH values. Thus, the curves for these amino acids in Fig. 1 intersect at about pH 3.25 and again at about pH 5.5.

Certain amino acids, such as leucine and isoleucine, which are difficult to separate by conventional paper chromatography show good separation on ion exchange papers, particularly at a high ionic strength in the pH range of 2.2 to 3.25. Phenylalanine and tyrosine, on the other hand, are more difficult to separate on ion exchange paper than on ordinary filter paper. Although they are affected to a somewhat different extent by changes in pH and ionic strength, it is difficult to obtain a clear-cut separation of the two amino acids in a mixture. The temperature, undoubtedly, plays an important role in ion exchange separations of amino acids. Thus, in column chromatography, tyrosine, phenylalanine, β-aminoisobutyric acid, and glucosamine appear together in the same fractions when eluted at 30° (12). When the temperature is raised to 50°, all four compounds are nicely separated (5, 6, 12). It is probable that similar improvements may be obtained by increasing the temperature in ion exchange paper chromatography.

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) XII

Preparation and Characterization of Trace Alkaloids

By GORDON H. SVOBODA, MARVIN GORMAN, ALBERT J. BARNES, Jr., and A. THORNTON OLIVER

The processing of this plant (1) for the four new oncolytic agents, vincaleukoblastine, leurosine, leurosidine, and leurocristine, has resulted in obtaining eight new companion dimeric alkaloids, these latter being obtained in yields of not more than 1.5×10^{-4} %. To date, a total of 24 new alkaloids have been reported from these laboratories. Procedural details and preliminary characterization of carosidine, carosine, pleurosine, neoleurosidine, vincarodine, catharicine, vindolidine, and neoleurocristine are described.

As a result of the search for the active antitumor principles contained in various alkaloidal fractions from this plant, 20 crystalline alkaloids, 16 of them being new, have been obtained in these laboratories[1] (2–6). Four of these, leurosine, vincaleukoblastine (VLB),[2] leurosidine, and leurocristine have demonstrated varying degrees of oncolytic activity in experimental tumors, the latter two being capable of producing "indefinite" survivors in DBA/2 mice infected with the P-1534 leukemia, a transplanted acute lymphocytic leukemia (7, 8). Clinically, VLB and leurocristine have elicited responses in human neoplasms, the former being commercially available as the sulfate[3] for the treatment of Hodgkin's disease and choriocarcinoma

The alkaloids obtained from this plant can be categorized into three groups. The first group consists of dimeric indole-indoline alkaloids of approximate composition of $C_{46}H_{52-62}N_4O_{8-10}$. Spectral studies (9) indicate a common structural relationship to the $C_{21}H_{24}N_2O_2$ indole, catharanthine, and the $C_{25}H_{32}N_2O_6$ indoline compound, vindoline, both of which are also obtained from this plant. The second group contains a number of miscellaneous smaller alkaloids ranging in molecular weight of 325–500. This group would include catharanthine and vindoline. A number of symmetrical dimeric alkaloids, primarily dihydroindoles, comprise the third group.

The eight alkaloids reported herein represent minor constituents obtained during the preparation of the four biologically active alkaloids mentioned above, and in no case were they found in greater amounts than 1.5×10^{-4} %. Five of these are members of the first group, while the remaining three, carosidine, vincarodine, and vindolidine, belong to the third class.

Carosidine was obtained by rechromatography of the residues remaining in the VLB sulfate mother liquors, while carosine and pleurosine were obtained by rechromatography of certain mixtures of leurosine and isoleurosine. Neoleurosidine resulted from rechromatography of impure leurosidine. The presence of this compound was suspected because of an abnormal pK'a ratio determined by electrometric titration.

Vincarodine was obtained by gradient pH extraction (6) of a fraction resulting from rechromatography of post-VLB column eluate, as were catharicine and vindolidine. Neoleurocristine was found by subjecting leurocristine sulfate mother liquors to the same type of gradient pH technique.

These new alkaloids are listed in Table I, which shows their empirical formulas, m.p.'s, pK'a's, specific rotations, and ultraviolet absorption maxima. Their infrared spectra are reproduced in Fig. 1 as an additional aid to their identification.

DISCUSSION

Carosidine.—Alumina chromatography of the bases obtained from the VLB sulfate mother liquors yielded this alkaloid by elution with chloroform-benzene (3:1). The ultraviolet spectrum is that of a typical substituted dihydroindole. The infrared spectrum (mull) shows the presence of

Received March 20, 1962, from the Organic Chemical Development and Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Accepted for publication April 3, 1962.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

The authors wish to thank the following individuals for their aid in the course of this investigation: Messrs. D. R. Bedwell, R. J. Armstrong, G. Johnson, and Miss R. Miller for laboratory assistance; Dr. R. Pfeiffer for crystallographic data; Dr. H. E. Boaz, Messrs. L. G. Howard, P. W. Landis, L. A. Spangle, Miss M. L. Hofmann, and Mrs. D. Stephens for physical data; Messrs. W. L. Brown, G. M. Maciak, H. L. Hunter, and R. Hughes for microanalysis.

[1] For the sake of brevity, experimental techniques repeated from earlier work are not described in detail. A reference is given to the previous paper.

[2] In compliance with the decision of the A.M.A. Council on Drugs, the name vincaleukoblastine should be replaced by vinblastine as the generic name for this alkaloid.

[3] Supplied as Velban, vinblastine sulfate, Lilly.

TABLE I.—NEW TRACE ALKALOIDS FROM *Vinca rosea* LINN.

Name	Formula[a]	M.P., °C.	$[\alpha]_D^{25}$ ($CHCl_3$)	pK'a in 33% DMF	U.V. $\lambda_{max.}^{EtOH}$, mμ
Carosidine	...	263–278, 283[b]	−89.8°	...	212, 254, 303
Carosine	$C_{46}H_{56}N_4O_{10}$	214–218	+6.0°	4.4, 5.5	255, 294
Pleurosine	$C_{46}H_{56}N_4O_{10}$	191–194[b]	+61.0°	4.4, 5.55	267, 308
Neoleurosidine	$C_{48}H_{62}N_4O_{11}$	219–225[b]	+41.6°	5.1	214, 268
Vincarodine	$C_{44}H_{52}N_4O_{10}$	253–256[b]	−197.4°	5.8 (66%)	230, 272, 298
Catharicine	$C_{46}H_{52}N_4O_{10}$	231–234[b]	+34.8°	5.3, 6.3	214, 268, 293, 315
Vindolidine	$C_{48}H_{64}N_4O_{10}$	244–250[b]	−113.2°	4.7, 5.3	261, 311
Neoleurocristine	$C_{46}H_{56}N_4O_{12}$	188–196[b]	−57.87°	4.68	220, 257, 298

[a] While these molecular formulas agree well with the analytical results for each particular alkaloid, it should be noted that they are to be considered as proximate at this time, in light of our experience with the other dimeric alkaloids (9). [b] With decomposition.

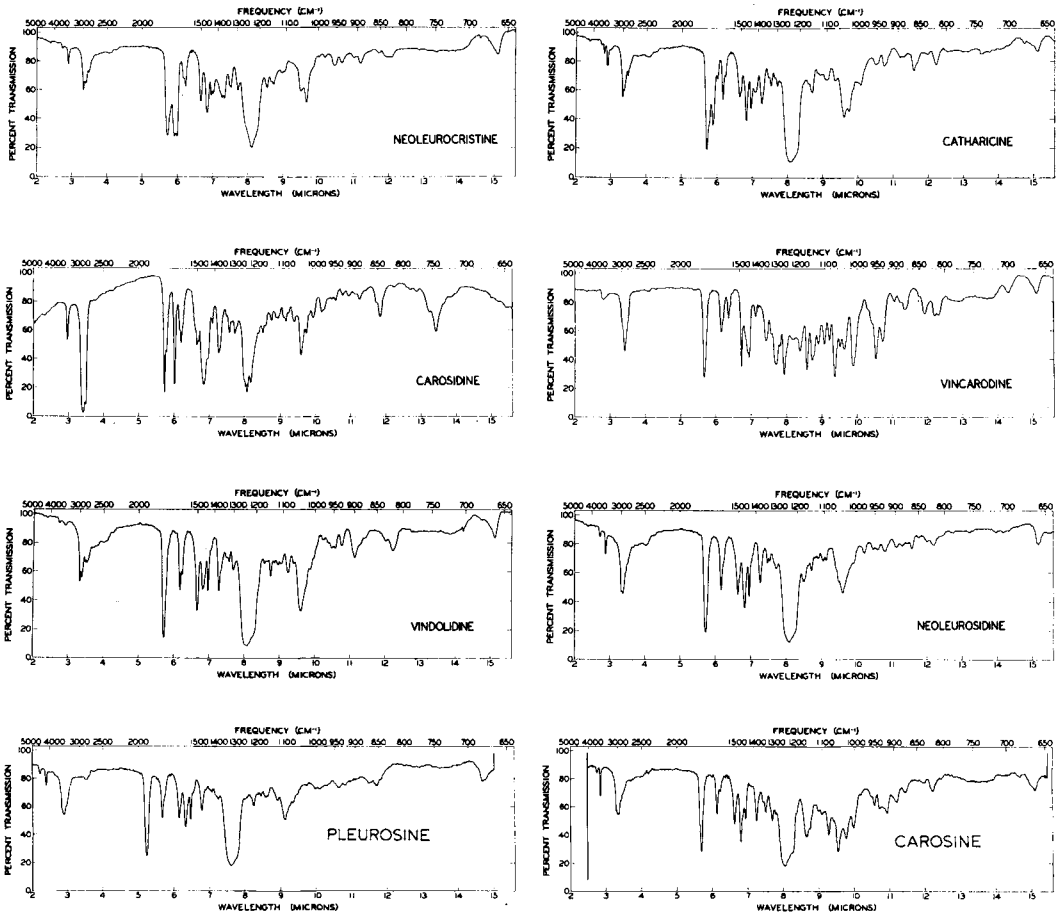


Fig. 1.—Infrared spectra of new alkaloids.

NH at 2.95 μ and carbonyl functions at 5.75 and 6.02 μ.

Carosine and Pleurosine.—Rechromatography of crude leurosine-isoleurosine mixtures described previously (5) yielded these two alkaloids, carosine from benzene-chloroform eluates (1:1) and pleurosine from chloroform.

The ultraviolet spectrum of carosine differs from those of the other dimeric alkaloids. Its infrared spectrum shows it to be a dimeric alkaloid containing hydroxyl, indole NH, and ester carbonyls. The NMR spectrum indicates the presence of two methyl esters, an aromatic methoxyl, an N-CH_3, and an O-acetyl group.

The ultraviolet spectrum of pleurosine is similar to those of the other dimeric alkaloids except that it does not shift in acid. The infrared spectrum resembles those of the other group 1 dimeric alkaloids.

Neoleurosidine.—The presence of this compound was suspected when an abnormal pK'a ratio was found to be associated with a particular crude

crystalline leurosidine sample. Leurosidine exhibits two pK'a groups at approximately 5.0 and 8.8, in equal mole per cent. The presence of neoleurosidine was thus indicated by an increased molar percentage of the lower pK'a. Electrometric titration of this sample, however, in 33% DMF gave the following data: 1.0 mole for pK'a 8.93 and 4.33 moles for pK'a 5.15. Rechromatography gave the new compound from the 1% methanol–99% chloroform eluate.

The ultraviolet spectrum is similar to those of the other dimeric alkaloids except that it does not shift to lower wavelength in acid as do the others. The infrared spectrum is consistent with those of the other related dimeric alkaloids of group 1 and contains the same type of hydroxyl as does leurosidine.

Vincarodine.—This alkaloid was obtained by chromatography of the bases from the VLB sulfate mother liquors and subjecting the benzene-chloroform (1:3) eluate to the gradient pH separation. It was found in the pH 4.40, 4.90, and 5.40 extracts.

The ultraviolet spectrum appears to be that of a substituted dihydroindole and closely resembles the alkaloid vincine from *Vinca minor* Linn. (10, 11). The infrared spectrum substantiates the relationship to vincine, showing the presence of a methyl ester and methoxyl substitution on the aromatic portion of the molecule, as well as an N-H.

Catharicine.—This new dimeric alkaloid was obtained by rechromatography of the chloroform-soluble post VLB eluate resulting from the commercial production of VLB sulfate (4) and by subjecting an early chloroform cut to gradient pH separation. It was found at pH 2.85 and 3.40.

Its ultraviolet spectrum is similar to those of the dimeric alkaloids which are composed of catharanthine-like and vindoline-like moieties, the slight bathochromic shift in acid, typical of the ultraviolet spectrum of leurosine, VLB, etc., is not found. The infrared spectrum is similar to that of VLB, but shows evidence of additional unsaturation which appears to be in the neighborhood of the basic nitrogen of the indole moiety (which titrates with a pK'a of 7.0 in VLB but does not in catharicine).

Vindolidine.—This alkaloid was obtained in the same manner as was catharicine, from the same starting material. The last two benzene-chloroform (1:3) fractions were treated with the gradient pH technique, and vindolidine was found to be present in the pH 3.90 and 4.40 extracts, the bulk of it being associated with the lower level. (Leurosidine was also obtained from these two fractions, but was found at pH levels of 5.40, 5.90, and 6.40.)

The ultraviolet spectrum is that of a typical substituted dihydroindole. The infrared spectrum supports a close structural relationship to vindolicine and vindoline and suggests the possibility that both this alkaloid and vindolicine are dimeric indoline alkaloids.

Neoleurocristine.—The presence of this alkaloid in the leurocristine sulfate mother liquors was demonstrated by paper chromatographic examination which indicated a less mobile material than leurocristine. Subjecting these mother liquors to gradient pH separation yielded the new alkaloid from the pH 3.40 fraction. The ultraviolet spectrum is quite similar to that of leurocristine, showing the anomalies to the other dimeric alkaloids that the latter does. The infrared spectrum contains an additional carbonyl band at 6.05μ, which may be due to an amide. As with leurocristine, no N-CH_3 is found in the NMR spectrum (1).

EXPERIMENTAL

Carosidine.—A quantity of VLB sulfate crude mother liquors was converted to the base, and 3.6 Kg. of this amorphous alkaloidal residue was chromatographed on 119 Kg. of alumina, deactivated in the usual manner (4). The column results are listed in Table II.

The crude methanol-crystallized carosidine obtained from fractions 32–35 was further purified by warming with 200 ml. of chloroform. The chloroform-insoluble material was filtered off and discarded. Concentration of the clear filtrate *in vacuo* to approximately 40 ml., followed by the addition of 400 ml. of acetone, with subsequent chilling produced 1.54 Gm. of the alkaloid in the form of blades having parallel extinction; gradual decompn. 263–278°, m.p. 283°, $[\alpha]_D^{26}$ −89.8° (c = 1, $CHCl_3$).

Anal.—Found: C, 67.75; H, 6.72; N, 8.22; O, 17.46.

Electrometric titration data were not available because of the insolubility of this alkaloid in the systems investigated. The solubility of this compound did not allow for calculation of extinction coefficients; $\lambda_{max.}^{EtOH}$ 212, 254, 303 mμ.

Carosine and Pleurosine.—Chromatography of a benzene solution containing 25 Gm. of a crude leurosine-isoleurosine mixture, corresponding to fractions 10 and 11 as described in an earlier paper (5), on 1000 Gm. of alumina, deactivated with 60

TABLE II.—CHROMATOGRAPHY OF BASES FROM VLB SULFATE MOTHER LIQUORS, YIELDING CAROSIDINE

Fraction	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1 (1500 L.)	Benzene	Oil	...	...
2–16 (3000 L.)	Benzene-chloroform (3:1)	Leurosine	3.8	Methanol
17–31 (3000 L.)	Benzene-chloroform (1:1)	Leurosine	43.3	Methanol
		VLB (as sulfate)	50.4	Ethanol
32–35 (600 L.)	Benzene-chloroform (1:3)	Carosidine	2.16	Methanol
36–41 (1500 L.)	Benzene-chloroform (1:3)	Amorphous residues	...	...
42–48 (1500 L.)	Chloroform	Amorphous residues	...	...

TABLE III.—CHROMATOGRAPHY OF CRUDE LEUROSINE-ISOLEUROSINE MIXTURES YIELDING CAROSINE AND PLEUROSINE

Fraction	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1 (1 L.)	Benzene	Oil	0.95	...
2–12 (6 L.)	Benzene	Isoleurosine	0.60	Methanol
13–14 (1.2 L.)	Benzene-chloroform (3:1)	Amorphous residues	0.35	...
15–21 (7 L.)	Benzene-chloroform (3:1)	Leurosine	3.5	Methanol
22–28 (12 L.)	Benzene-chloroform (3:1)	Amorphous residues	1.75	...
29–41 (20 L.)	Benzene-chloroform (1:1)	Amorphous residues	3.5	...
42–45 (8 L.)	Benzene-chloroform (1:1)	Carosine	0.300	Methanol
46–48 (6 L.)	Benzene-chloroform (1:1)	Amorphous residues	0.12	...
49–55 (14 L.)	Chloroform	Pleurosine	0.225	Methanol

TABLE IV.— CHROMATOGRAPHY OF IMPURE LEUROSIDINE, YIELDING NEOLEUROSIDINE

Fraction	Eluting Solvent	Compound	Wt., Gm.	Crystallizing Solvent
1 (2400 ml.)	Benzene	Oil	0.01	...
2 (2400 ml.)	Benzene-chloroform (3:1)	Oil	0.01	...
3–21 (2600 ml.)	Benzene-chloroform (1:1)	Amorphous residues	0.91	...
22–26 (500 ml.)	Benzene-chloroform (1:3)	Amorphous residues	0.42	...
27–46 (2000 ml.)	Benzene-chloroform (1:3)	Leurosidine	0.5875	Methanol
47–73 (3880 ml.)	Chloroform	Leurosidine	0.5265	Methanol
74 (500 ml.)	Chloroform-methanol (99:1)	Amorphous residue	0.04	...
75–86 (1410 ml.)	Chloroform-methanol (99:1)	Neoleurosidine	1.2725	Methanol
87–90 (360 ml.)	Chloroform-methanol (19:1)	Neoleurosidine	0.007	Methanol
91–101 (1280 ml.)	Chloroform-methanol (19:1)	Amorphous residues	0.40	...
102 (1000 ml.)	Methanol	Amorphous residues	0.33	...

ml. of 5% acetic acid gave the results as listed in Table III.

Carosine.—Recrystallization of the crude crystalline base from methylene chloride-ether produced 0.240 Gm. of the base as needles with parallel extinction; m.p. 214–218°; $[\alpha]_D^{26}$ +6.0° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{56}N_4O_{10}$: C, 66.97; H, 6.84; N, 6.79; O, 19.39. Found: C, 66.71, 66.71; H, 7.02, 6.88; N, 6.52; O, 19.61.

pK'a 4.4, 5.5, electrometric titration, 33% DMF; mol. wt., 934; $\lambda_{max.}^{EtOH}$ 255 mμ [log E(1%, 1 cm.) 2.21], 294 mμ [log E(1%, 1 cm.) 2.19].[4]

Pleurosine.—Recrystallization of the base from methanol yielded 0.175 Gm. of long blades having parallel extinction; m.p. 191–194° (decompn.); $[\alpha]_D^{26}$ −61.0° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{56}N_4O_{10}$: C, 66.97; H, 6.84; N, 6.79; O, 19.39. Found: C, 67.04; H, 7.08; N, 6.49; O, 19.44.

pK'a 4.4, 5.55, electrometric titration, 33% DMF; mol. wt., 908; $\lambda_{max.}^{EtOH}$ 267 mμ [log E(1%, 1 cm.) 2.32], 308 mμ [log E(1%, 1 cm.) 1.87].

Neoleurosidine.—A benzene solution of 8.5 Gm. of impure leurosidine, exhibiting the abnormal pK'a ratio described above, was chromatographed on 343 Gm. of alumina (Alcoa, Grade F-20) deactivated with 11.5 ml. of 10% acetic acid in the usual manner (see Table IV).

The crude methanol-crystallized neoleurosidine from fractions 75–90 were combined and were recrystallized from methanol, yielding 1.017 Gm. of pure alkaloid in the form of rods with inclined extinction; m.p. 219–225° (decompn.); $[\alpha]_D^{26}$ + 41.6° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{48}H_{62}N_4O_{11}$: C, 66.18; H, 7.17; N, 6.43; O, 20.20. Found: C, 66.20; H, 7.26; N, 6.49; O, 20.14; OCH_3, 13.4; OAC, 8.65; (C)-CH_3, 4.21.

pK'a 5.1, electrometric titration, 33% DMF; mol. wt., 452 × 2 = 904; $\lambda_{max.}^{EtOH}$ 214 mμ [log E(1%, 1 cm.) 2.77], 268 mμ [log E(1%, 1 cm.) 2.29]; shoulders at 285 mμ [log E(1%, 1 cm.) 2.19], 295 mμ [log E (1%, 1 cm.) 2.12], 310 mμ [log E(1%, 1 cm.) 1.83].

Vincarodine.—The gradient pH technique (6) used to prepare this alkaloid from the benzene-chloroform (1:3) eluate of columned bases from the VLB sulfate mother liquors is as follows: a total of 182 Gm. of amorphous base was dissolved in 7 L. of benzene and filtered. The clear solution was mixed with 9 L. of 0.1 *M* citric acid and the mixture was distilled *in vacuo* to remove the benzene. The aqueous phase was filtered and subsequently extracted with one 9-L. portion of benzene at each of the following pH levels: 3.2, 3.9, 4.4, 4.9, 5.4, 5.9, and 8.0, the pH being raised after each extraction by the addition of 28% ammonia.

The crude cuts from pH 4.4, 4.9, and 5.4 (40.4 Gm.) crystallized from methanol, yielding 3.0 Gm. of crude crystalline vincarodine. Recrystallization from chloroform-methanol yielded 2.55 Gm. of base as blades having parallel extinction; m.p. 253–256° (decompn.); $[\alpha]_D^{26}$ −197.4° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{44}H_{52}N_4O_{10}$: C, 66.31; H, 6.58; N, 7.03; O, 20.08. Found: C, 66.56; H, 6.62; N, 7.20; O, 20.12.

pK'a 5.8, electrometric titration, 66% DMF; mol. wt., 400 if monomeric, 800 if dimeric. The molecular weight by vapor pressure lowering indicated that the dimeric formulation was correct.

[4] Because of the high degree of solvation of these molecules, the samples taken for pK'a determination and ultraviolet spectral measurements at times did not give the same molecular weight as those specially dried for analysis. Therefore, we report the ultraviolet absorption intensities as log $E_{1\%}$ rather than as log ϵ_M.

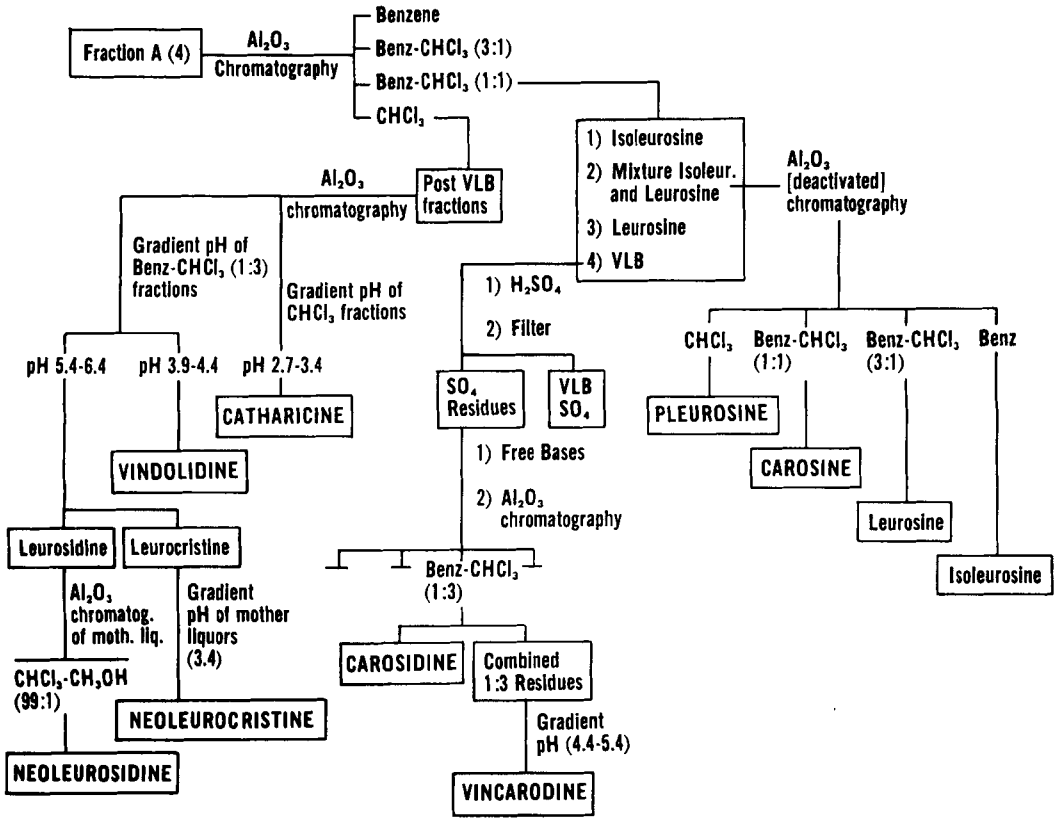


Fig. 2.—Flow diagram.

$\lambda_{max.}^{EtOH}$ 230 mμ [log E(1%, 1 cm.) 2.82], 272 mμ [log E(1%, 1 cm.) 2.30], 298 mμ [log E(1%, 1 cm.) 2.04].

Catharicine.—Rechromatography of the chloroform-soluble post-VLB eluate from fraction 1 and subjecting 256.55 Gm. of an early chloroform fraction, corresponding to fraction 47 as described in (6), to the gradient pH technique previously recorded, yielded this dimeric indoline alkaloid from pH cuts 2.70 and 3.40. The amorphous residues from each cut readily crystallized from acetone, yielding 1.1805 and 4.535 Gm. of crude crystalline base, respectively. Recrystallization from acetone, or from any of the commonly-used solvents, was ineffectual, always producing a gel. It was necessary to rechromatograph the base to obtain a pure entity.

The pure base crystallizes from acetone as irregular plates; m.p. 231–234° (decompn.), $[\alpha]_D^{26}$ + 34.8° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{52}N_4O_{10}$: C, 66.97; H 6.84; N, 6.79; O, 19.39. Found: C, 66.20; H, 6.70; N, 6.55; O, 20.00.

pK'a 5.3, 6.3, electrometric titration, 33% DMF; mol. wt., 894; $\lambda_{max.}^{EtOH}$ 214 mμ [log E(1%, 1 cm.) 2.81], 268 mμ [log E(1%, 1 cm.) 2.29], 293 mμ [log E(1%, 1 cm.) 2.12], 315 mμ [log E(1%, 1 cm.) 1.74]; shoulder at 283 mμ [log E(1%, 1 cm.) 2.23].

Vindolidine.—Rechromatography of the chloroform-soluble post-VLB eluate from fraction 1 and subjecting 69.25 Gm. of the late chloroform-benzene (3:1) fractions, corresponding to fractions 43 and 44 as described in (6) to gradient pH separation gave 0.642 Gm. of crude crystalline vindolidine from pH cut 3.92 and 0.037 Gm. from pH cut 4.42. Recrystallization from methanol yielded microcrystals; m.p. 244–250° (decompn.); $[\alpha]_D^{26}$ −113.2° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{48}H_{64}N_4O_{10}$: C, 64.84; H, 7.26; N, 6.30; O, 21.60. Found: C, 64.90, H, 7.22; N, 6.56; O, 21.56; OCH_3, 17.45; (N)CH_3, 3.38; (C)CH_3, 4.94; OAC, 4.52.

pK'a 4.7, 5.3, electrometric titration, 33% DMF; mol. wt., 880; $\lambda_{max.}^{EtOH}$ 261 mμ [log E(1%, 1 cm.) 2.33], 311 mμ [log E(1%, 1 cm.) 2.12].

Neoleurocristine.—Subjecting 4.786 Gm. of leurocristine sulfate crude mother liquors to gradient pH separation in the usual manner resulted in obtaining 0.181 Gm. of this new alkaloid from the 0.40 Gm. of crude amorphous residue in the pH 3.40 cut by crystallization from methanol, from which it crystallizes as irregular plates; m.p. 188–196° (decompn.); $[\alpha]_D^{26}$ −57.87° (c = 1, $CHCl_3$).

Anal.—Calcd. for $C_{46}H_{56}N_4O_{12}$: C, 64.47; H, 6.59; N, 6.54; O, 22.41. Found: C, 64.62, 64.82; H, 6.55, 6.72; N, 6.57, 6.34; O, 22.05; OCH_3, 10.36; (C)CH_3, 4.50; OAc, 4.42.

pK'a 4.68, electrometric titration, 33% DMF; mol. wt., 827; $\lambda_{max.}^{EtOH}$ 220 mμ [log E(1%, 1 cm.) 2.74], 257 mμ [log E(1%, 1 cm.) 2.23], 298 mμ [log E(1%, 1 cm.) 2.29]; shoulders at 264 mμ [log E(1%, 1 cm.) 2.23], 290 mμ [log E(1%, 1 cm.) 2.24].

A flow diagram illustrating the manner in which these alkaloids were obtained is shown in Fig. 2.

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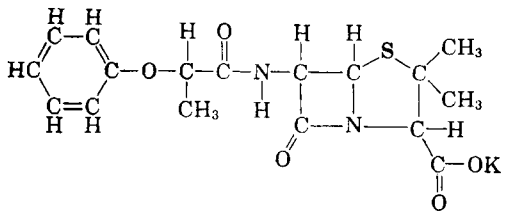
Stability of Potassium Phenethicillin I

Kinetics of Degradation in Aqueous Solution

By M. A. SCHWARTZ, A. P. GRANATEK, and F. H. BUCKWALTER

Kinetic studies of the degradation of potassium phenethicillin in aqueous solution have been carried out and presented with a view toward stability prediction. Phenethicillin, an acid with pKa 2.9, is hydrolyzed by both acid and base with minimum rate of degradation at about pH 6.5 at 35°. In acid solution the ionic form of phenethicillin is hydrolyzed about 13 times as fast as the free acid. The $HPO_4^=$ ion was found to be a general base catalyst. The effect of temperature in the neutral pH region has been determined, and from this data the aqueous solution stability of potassium phenethicillin at 25 and 4° has been predicted.

Potassium phenethicillin[1] is the first commercially produced semisynthetic penicillin.



Its chemical, microbiological and pharmacological properties have been described (1–3). Clinical studies have also been reported (4, 5). The present work was undertaken to enable the prediction of the optimum stability formulating conditions for potassium phenethicillin in aqueous solution.

EXPERIMENTAL

One lot of commercially produced potassium phenethicillin containing approximately 60% of the L-form was used throughout this study. All buffer materials were reagent grade.

The kinetic studies were carried out as follows: 90 ml. of buffer (pH range given in Table I) in a 100-ml. volumetric flask was brought to bath temperature and then 1.0 ml. of a freshly prepared 0.1 *M* aqueous solution of potassium phenethicillin was added with mixing. At appropriate intervals, 2.0-ml. samples of the reaction mixture were taken and added to 8.0 ml. of cold 0.5 *M* pH 6 citrate-phosphate buffer to quench the reaction. These solutions were immediately frozen and kept in this state until just before assay. The phenethicillin potency of these samples was determined by the microbiological turbidimetric assay method with *Staph. aureus* strain FDA209-P as the inoculum.

In order to determine the effect of freezing the quenched samples until assay, a solution of 80 mcg./ml. of potassium phenethicillin in the strong pH 6 buffer was divided into 10-ml. samples and frozen. One sample was assayed each day for 13 days. No significant loss of potency was observed despite a day-to-day variability of 14.5%. To avoid this, all samples from a single kinetic run were assayed on the same day. The maximum time these were kept frozen was 6 days.

The ionic strength of all the buffers was adjusted to 0.5 with potassium chloride. The pH was determined with a Beckman Zeromatic pH meter using glass and calomel electrodes.

The pKa of phenethicillin was determined in 0.5 *M* potassium chloride by taking the pH of a half-neutralized 0.01 *M* solution.

Received August 14, 1961, from the Research Division, Bristol Laboratories, Syracuse, N. Y.
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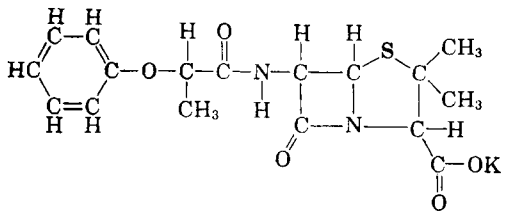
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The kinetic studies were carried out as follows: 90 ml. of buffer (pH range given in Table I) in a 100-ml. volumetric flask was brought to bath temperature and then 1.0 ml. of a freshly prepared 0.1 *M* aqueous solution of potassium phenethicillin was added with mixing. At appropriate intervals, 2.0-ml. samples of the reaction mixture were taken and added to 8.0 ml. of cold 0.5 *M* pH 6 citrate-phosphate buffer to quench the reaction. These solutions were immediately frozen and kept in this state until just before assay. The phenethicillin potency of these samples was determined by the microbiological turbidimetric assay method with *Staph. aureus* strain FDA209-P as the inoculum.

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TABLE I.—KINETIC STUDIES OF THE DEGRADATION OF POTASSIUM PHENETHICILLIN

Run No.	Temp., °C.	pH	Buffer Ingredients[a] HCl	H_3PO_4	$H_2PO_4^-$	$HPO_4^=$	$H_3Cit.$	$H_2Cit.^-$	$HCit.^=$	$Cit.^{\equiv}$	NaOH	k' ($hr.^{-1}$)[b]	$\sigma k'$
1	35	1.0	0.1	...	...	...	...	...	...	...	...	1.55	0.07
2	35	1.5	0.032	...	...	...	...	...	...	...	...	0.602	0.06
3	35	2.0	...	0.048	0.052	...	...	...	...	...	...	0.256	0.01
4	35	2.3	...	0.007	0.001	...	0.082	0.014	0.0004	...	...	0.219	0.001
5	35	2.6	...	0.004	0.018	...	0.067	0.022	0.0002	...	...	0.158	0.011
6	35	2.9	...	0.0033	0.038	...	0.043	0.036	0.001	...	...	0.109	0.004
7	35	3.1	...	0.0031	0.042	...	0.037	0.039	0.0009	...	...	0.0798	0.0006
8	35	3.3	...	0.002	0.055	...	0.022	0.047	0.002	...	...	0.0644	0.0003
9	35	3.9	...	0.0007	0.076	...	0.006	0.047	0.008	...	...	0.0179	0.0002
10	35	5.0	...	...	0.101	0.002	...	0.014	0.025	0.010	...	0.00480[c]	0.001
11	35	5.6	...	...	0.107	0.0086	...	0.002	0.015	0.024	...	0.00485	0.0005
12	35	6.0	...	...	0.105	0.021	...	...	0.007	0.029	...	0.00528	0.0005
13	35	6.7	...	...	0.045	0.055	...	...	...	...	...	0.00857	0.0002
14	35	6.7	...	...	0.0225	0.0275	...	...	...	...	...	0.00635	0.0002
15	35	7.4	...	...	0.0142	0.0858	...	...	...	...	...	0.0118	0.0001
16	35	7.4	...	...	0.0071	0.0429	...	...	...	...	...	0.00668	0.0002
17	35	8.0	...	...	0.0048	0.0952	...	...	...	...	...	0.0141	0.0002
18	35	11.15[d]	...	...	...	...	...	...	...	...	0.002[e]	787[f]	...
19	35	10.85[d]	...	...	...	...	...	...	...	...	0.001[e]	758[f]	...
20	35	11.40[d]	...	...	...	...	...	...	...	...	0.003[e]	731[f]	...
21	55	6.7	...	...	0.045	0.055	...	...	...	...	...	0.0452	0.001
22	65	6.7	...	...	0.045	0.055	...	...	...	...	...	0.113	0.005

[a] Approximate molar concentrations, calculated from the following pKa: H_3PO_4 = 1.96, $H_2PO_4^-$ = 6.70, $H_3Cit.$ = 3.08, $H_2Cit.^-$ = 4.75, $HCit.^=$ = 5.40. [b] Observed first-order constant. [c] Extrapolated value. [d] Initial pH. [e] Initial Concentration. [f] Second-order rate constant (L. $mole^{-1}$ $hr.^{-1}$).

RESULTS AND DISCUSSION

Order of Reaction.—At constant temperature and pH the hydrolysis of phenethicillin is an overall first-order reaction as shown by the linear log concentration-time plots of Fig. 1. These are typical of the results obtained throughout this study.

Runs 18 through 20 in Table I were made using 0.001 *M* potassium phenethicillin with 0.001, 0.002, and 0.003 *M* hydroxyl ion. The data are plotted as second-order reactions as in Fig. 2. The rate constants were determined from the slopes by the method of least squares.

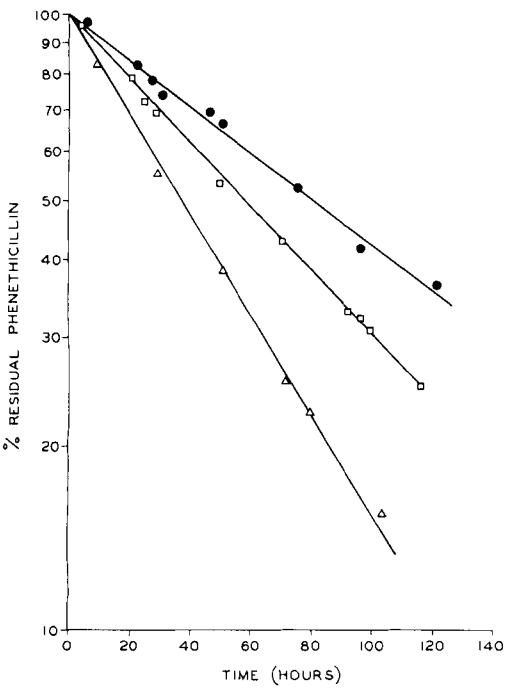


Fig. 1.—First-order plots of degradation of phenethicillin at 35° at several pH's. Δ, pH 3.9; ● pH, 6.7; □, pH 7.4.

Table I lists the buffers used and the observed results. The k' values were all calculated by the method of least squares. The standard deviations, $\sigma k'$, were obtained essentially by the method described by McLeod, *et al.* (6).

pH Dependence.—Figure 3 shows the dependence of the rate of hydrolysis of phenethicillin on pH. The dashed curve shows the actual values found while the solid curve is corrected for buffer effects. The effect of buffers is discussed in a subsequent section.

The curvature on the acid side indicates that more than one species is involved in the reaction. Since phenethicillin is an acid with pKa 2.9, one may calculate the proportions of unionized and ionized material as a function of pH from the following relationships

$$f_{HP} = \frac{[H^+]}{[H^+] + Ka} \text{ and } f_{P^-} = \frac{Ka}{[H^+] + Ka} \quad \text{(Eq. 1)}$$

where f_{HP} = fraction of phenethicillin as the free acid [HP] and f_{P^-} = fraction of phenethicillin ionized [P^-].

The overall hydrogen ion catalyzed reaction may be written in the form

$$-\frac{dP_T}{dt} = k_1 [H^+] [HP] + k_2 [H^+] [P^-] = k' P_T \quad \text{(Eq. 2)}$$

where k_1 and k_2 are the rate constants for the hydrolysis of acid and ion, respectively, P_T is the total drug concentration, and k' is the observed pseudo first-order rate constant. Dividing through by $P_T[H^+]$

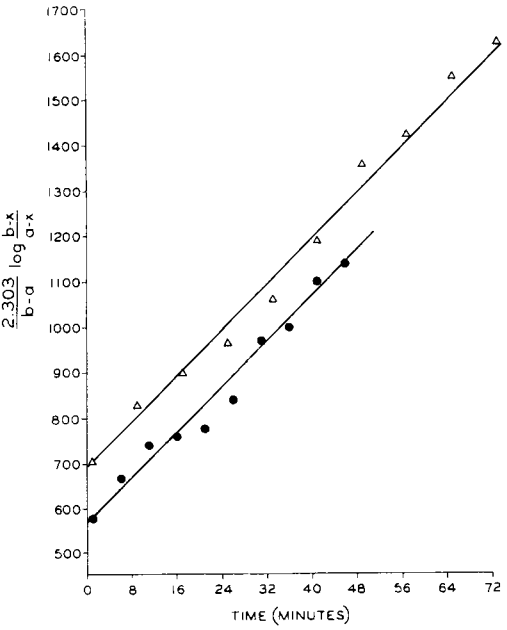


Fig. 2.—Second-order plots of reaction of phenethicillin with hydroxyl ion at 35°. a = Initial molar concentration of phenethicillin, b = initial molar concentration of $[OH^-]$. ●, b = 0.003, a = 0.001; Δ, b = 0.002, a = 0.001.

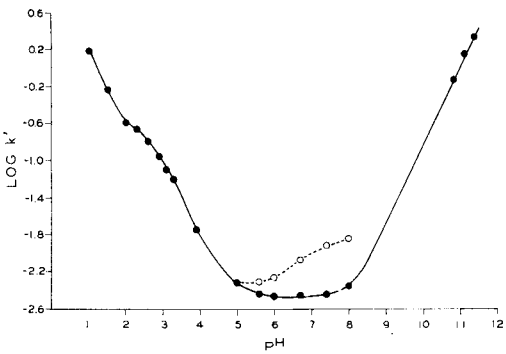


Fig. 3.—pH dependence of rate of hydrolysis of phenethicillin at 35°.

$$\frac{k'}{[H+]} = k_1 f_{HP} + k_2 f_{P^-} \qquad \text{(Eq. 3)}$$

Since $f_{HP} + f_{P^-} = 1$, we may rearrange Eq. (3) to

$$\frac{k'}{[H^+]} = (k_1 - k_2) f_{HP} + k_2 \qquad \text{(Eq. 4)}$$

A plot of $k'/[H^+]$ as a function of f_{HP} should give a straight line with slope $k_1 - k_2$ and intercept k_2. Such a plot is shown in Fig. 4. The constants derived from the best fit line as determined by the method of least squares are[2]: k_1 = 12.5 ± 5.7 L. mole^{-1} hr.$^{-1}$ and k_2 = 162 ± 6.5 L. mole^{-1} hr.$^{-1}$ at 35°. The greater affinity of the negatively charged ion for proton probably accounts for its faster reaction rate.

[2] The errors noted here and subsequently in this paper represent the standard deviation.

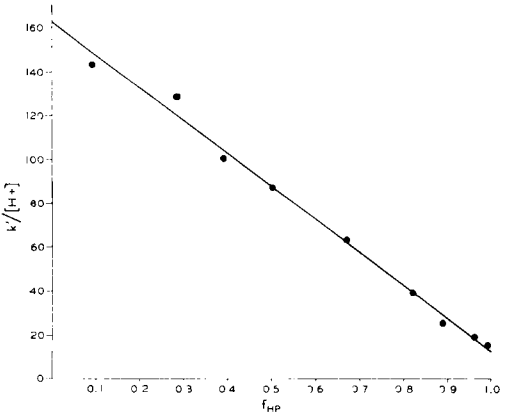


Fig. 4.—Plot of Eq. 4 from which was determined k_1 and k_2 at 35°.

The second-order constant for alkaline hydrolysis was determined directly to be 758 ± 28 L. mole^{-1} hr.$^{-1}$.

From these values the pH of minimum degradation rate may be determined as follows

$$k' = k_2 [H^+] + k_{OH^-} [OH^-] = k_2 [H^+] + \frac{K_w k_{OH^-}}{[H^+]} \qquad \text{(Eq. 5)}$$

since all the phenethicillin is in the ionic form in this pH region. Differentiating k' with respect to $[H^+]$, we obtain

$$\frac{dk'}{d[H^+]} = k_2 - \frac{K_w k_{OH^-}}{[H^+]^2} = 0 \qquad \text{(Eq. 6a)}$$

$$[H^+]_{min.} = \sqrt{\frac{k_{OH^-} K_w}{k_2}} \qquad \text{(Eq. 6b)}$$

At 35° this results in $pH_{min.}$ = 6.5. This, incidentally, is the same value observed in the case of benzyl penicillin (7).

Effect of Buffers.—In the pH range 5.5 to 7.0 where specific acid and base effects were calculated to be negligible, appreciable rates of degradation were observed. This led to the conclusion that water and/or one or more of the buffer ingredients were catalytic species. Examination of the results indicated that the $HPO_4^=$ ion was a general base catalyst. This effect may be formulated as follows

$$k' = k_2[H^+] + k_{OH^-}[OH^-] + k_0 + k_{HPO_4^=} [HPO_4^=] \qquad \text{(Eq. 7a)}$$

which when transposed becomes

$$k' - k_2[H^+] - k_{OH^-}[OH^-] = k'' = k_0 + k_{HPO_4^=} [HPO_4^=] \qquad \text{(Eq. 7b)}$$

A plot of k'' against $HPO_4^=$ should therefore be linear with slope $k_{HPO_4^=}$ and intercept k_0, where k_0 represents the rate of reaction with H_2O. Figure 5 is such a plot, and by the method of least squares the 35° values of k_0 = 0.00325 ± 0.0001 and $k_{HPO_4^=}$ = 0.094 ± 0.003 were obtained. Unprotonated citrate ion appears to have no catalytic effect on the reaction rate.

Effect of Temperature.—For purposes of stability prediction the rate of reaction was run at pH 6.7 as a function of temperature. The Arrhenius plot

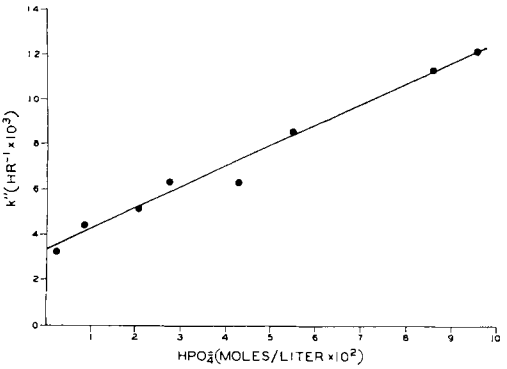


Fig. 5.—Plot showing catalytic effect of $HPO_4^=$ ion on rate of hydrolysis of phenethicillin at 35°

from these data is shown in Figure 6. The energy of activation was calculated to be 17.6 ± 0.3 Kcal. per mole.

Prediction of Stability.—For a solution of phenethicillin not containing phosphate the rate of degradation at room and refrigerator temperatures may be calculated from k_0 determined at 35° and the heat of activation at pH 6.7. This assumes that the latter is the same as the heat of activation for the reaction with water.[3] In this way the following values were calculated k_0 = 0.00134 hr.$^{-1}$ at 25° (room temperature) and k_0 = 0.000129 hr.$^{-1}$ at 4° (refrigerator temperature).

Further application of this data to product formulation will be considered in a subsequent paper.

SUMMARY AND CONCLUSIONS

Degradation rates for potassium phenethicillin have been determined over the pH range 1–11 and as a function of temperature in the neutral region. On the acid side, both the free acid and ionized species undergo degradation at different rates with the constant for the ion being about 13 times that of the acid. The half-life of potassium phenethicillin at 35° and pH 1.5 is about 1 hour. A comparable value for penicillin G is about 4 minutes (7). This fifteenfold increase in potassium phenethicillin stability over penicillin G at pH 1.5 and 35° may account for the greater serum levels achieved with oral administration of potassium phenethicillin.

The pH of the minimum rate of degradation of potassium phenethicillin is 6.5, although the rate of degradation does not increase appreciably until the pH is brought below 5.5 or above 7. Secondary phosphate ion catalyzes the hydrolysis while unprotonated citrate ion does not have a catalytic effect. Optimum aqueous solution stability conditions for potassium phenethicillin would therefore be provided by maintaining the pH of the preparation between 6 and 6.5 using a buffer such as citrate which has no effect on its hydrolysis in this pH range. Applications of these data to product formulation are considered in a subsequent paper (8).

[3] This was found to be true as will be shown in a subsequent paper.

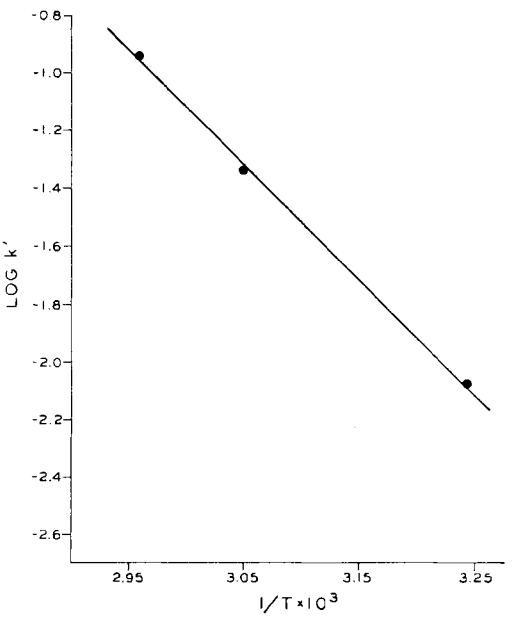


Fig. 6.—Arrhenius plot for temperature dependence of rate of hydrolysis of phenethicillin at pH 6.7.

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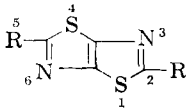
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Derivatives of Thiazolo[5,4-d]thiazole

By HIKMAT T. FIKRAT and J. F. ONETO

A series of bis-2,5-substituted thiazolo[5,4-d]thiazoles has been prepared and examined for biological activity.

THE CONDENSATION of dithio-oxamide with aromatic aldehydes was first described by Ephraim (1). Recently, Johnson and Ketcham (2) reported on studies concerning this reaction and established the structure of the resulting parent heterocycle as thiazolo[5,4-d]thiazole, I, with substituents at the two and five positions being equivalent.



I

The purpose of our investigation was to prepare a number of hitherto undescribed thiazolothiazole derivatives for biological examination. Compounds of this class were considered to be of empirical interest because of the included thiazole moiety which occurs in a wide variety of agents exhibiting diverse biological activities.

The synthesis was effected in low to moderate yields (10–88%) through condensation of dithio-oxamide with appropriate aldehydes following essentially the published procedure (2). Of particular interest was the development of an alternate preparative method which was studied in the preparation of the previously described benzaldehyde derivative (2), and the pyridine-3-aldehyde and 2,2-dimethyl-4-cyanobutyraldehyde derivatives (Table I). The condensation was carried out in refluxing pyridine and indicated that in the above instances high reaction temperatures were not essential for successful results. However, the yields obtained by this approach were invariably lower than those realized from the published procedure.

Received May 1, 1961, from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Accepted for publication July 28, 1961.
This paper represents part of a dissertation submitted by Hikmat T. Fikrat in partial fulfillment of the requirements for the Ph.D. degree, 1959.
Portions of the paper are adapted from the honorable-mention winning manuscript submitted by Hikmat T. Fikrat in the Western Region, 1959, Lunsford-Richardson Pharmacy Award competition.
The authors are indebted to Dr. L. A. Strait and Mr. M. Hrenoff for the spectral analyses and interpretations. They also wish to express their gratitude to Dr. Leonard Daub and his staff, Parke Davis and Co., for the antimicrobial evaluations, and to Dr. Roger Ketcham for his continued interest and valuable counsel. The pharmacological study of compound 14 was carried out with the cooperation of Dr. Frederick Meyers, Department of Pharmacology, University of California Medical Center, San Francisco.

The apparent limitation of the reaction to aromatic type aldehydes has been reported (2). Our investigation of a number of saturated and α,β-unsaturated aliphatic aldehydes, in addition to the aldehydes studies by Johnson and Ketcham, supports this observation with the exception of the previously mentioned 2,2-dimethyl-4-cyanobutyraldehyde. As this cyano aliphatic aldehyde is devoid of α hydrogen, it was of interest to investigate the reaction behavior of the analogous hydroxypivaldehyde-(2,2-dimethyl-3-hydroxypropionaldehyde) and its acetyl and benzoyl derivatives. Efforts to obtain the corresponding thiazolothiazole derivatives from these aldehydes were unsuccessful.

The reaction of quinoline-2-aldehyde with dithio-oxamide yielded an acid-soluble compound melting at 109–111°, and an acid-insoluble compound which melted over a wide range (230–265°) after several recrystallizations from chloroform. Neither of the products proved to be thiazolothiazole derivatives.

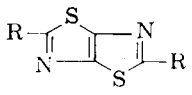
During the course of the investigation, the diamide, dihydrazide, and diisopropylidenehydrazide derivatives of thiazolothiazole-2,5-dicarboxylic acid were prepared and studied.

Inasmuch as bis-(1,1-dimethyl-3-cyanopropyl)-thiazolo[5,4-d]thiazole is the only compound to have been prepared from an aliphatic aldehyde, its structure in addition to analyses was further confirmed by comparing its ultraviolet and infrared spectra with those reported for the parent heterocycle (3).

The ultraviolet absorption of the compound was measured with a Cary model 11 spectrometer at a concentration of 2.5 mg. % in 95% ethanol. Bands appearing at 244 mμ (log ϵ = 3.85) and 251 mμ (log ϵ = 4.11) correspond in shape and contour with the reported thiazolothiazole bands at 242 and 255 mμ (log ϵ = 3.94). A strong band appearing at 278 mμ corresponds with the 260 mμ (log ϵ = 3.87) of thiazolothiazole.

The infrared absorption of the compound was measured in a potassium bromide disk (3 mg. per 400 mg. KBr) with a Perkin-Elmer model 21 spectrophotometer. The observed characteristic vibrational frequencies lead to the following assignments: a broad unresolved band at 3.41 μ in the C—H stretching region is understandable due to the increased variety of C—H bonds as compared with the sharp 3.31 μ band due to the

TABLE I.—BIS-2,5-SUBSTITUTED THIAZOLO[5,4-d]THIAZOLES



No.[a]	R	R—CHO Ratio[b]	Time,[c] Min.	Intern. Temp.,[c] °C.	Yield, %	M.p.,[d] °C.	Formula	Analyses,[e] % Nitrogen Calcd.	Nitrogen Found	Sulfur Calcd.	Sulfur Found
1	2-Pyridyl	8	30	160–170	51	325–326	$C_{14}H_8N_4S_2$	...	...	21.63	21.46
2	3-Pyridyl	8	30	165–175	59	268–269	$C_{14}H_8N_4S_2$	18.95	19.11	...	...
		(8)			(38)[f]						
3	4-Pyridyl	8	30	160–165	85	300–302	$C_{14}H_8N_4S_2$	18.95	19.07	...	...
4	6-Methyl-2-pyridyl	10	20	170–200	56	299–302	$C_{16}H_{12}N_4S_2$	17.26	17.50	19.77	19.48
5	2-Carboxyphenyl	10	15	220–230	10	302–305	$C_{18}H_{10}N_2O_4S_2$	7.32	7.48	16.79	16.52
6	2,4-Dimethoxyphenyl	5	25	180–200	58	291–293	$C_{20}H_{18}N_2O_4S_2$	6.76	6.94	15.47	15.45
7	3,4-Dimethoxyphenyl	8	30	195–200	64	269–272	$C_{20}H_{18}N_2O_4S_2$	6.76	6.74	15.47	15.25
8	3-Methoxy-4-hydroxyphenyl	8	30	180–200	52	270–273	$C_{18}H_{14}N_2O_4S_2$	7.32	7.47	16.77	16.56
9	4-Acetamidophenyl[g]	7.5	5	190–200	73	388–396	...	...	...	...	...
10	2-Hydroxy-1-naphthyl	5	20	170–200	54	338–342	$C_{24}H_{14}N_2O_2S_2$	6.57	6.72	15.04	14.86
11	2-Thienyl	5	20	170–180	88	238–240	$C_{12}H_6N_2S_4$	9.14	9.05	41.85	41.62
12	3-Indolyl	5	30	175–180	31	>400	$C_{20}H_{12}N_4S_2$	15.07	15.32	17.22	17.01
13	4-Quinolyl	10	5[h]	230	40	254–256	$C_{22}H_{12}N_4S_2$	14.14	13.85	...	...
14	1,1-Dimethyl-3-cyanopropyl[i]	5	20	200	39	109–111	$C_{16}H_{20}N_4S_2$	...	...	19.29	19.09
		(8)			(27)[f]						

[a] Compounds 1, 2, 3, 4, 7, and 12 were recrystallized from pyridine; 5 from ethanol; 6 and 8 from phenol-ethanol; 10 from pyridine or benzene-alcohol; 11 from pyridine, chloroform or benzene; 13 from chloroform, and 14 from acetone, benzene, ethanol, or ether. [b] Molar ratio of aldehyde to dithio-oxamide. [c] Time of heating during the indicated internal temperature range. [d] Melting points were determined on a Nalge m.p. apparatus and are uncorrected. [e] Analyses were performed at the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. [f] Procedure B. [g] The crude product was hydrolyzed with hydrochloric acid in butanol to the bis-4-aminophenyl derivative; m.p. 296–300° [Lit. (2) 301–303°]. [h] The reaction mixture was then cooled to 180–200° and maintained at that temperature range for 20 minutes. [i] The 2,2-dimethyl-4-cyanobutyraldehyde was supplied through the courtesy of Eastman Chemical Products, Inc., Kingsport, Tenn.

C—H stretch in thiazolothiazole. The presence of the nitrile group is indicated by a characteristic stretching band at 4.5 μ. Peaks at 6.7, 6.8, and 6.9 μ can be assigned to the $-CH_2-$ and $-CH_3$ deformation vibrations. Peaks at 7.14 and 7.26 μ are assigned to the deformation vibrations of the $=C(CH_3)_2$ grouping. The assigned structure appears to be in general agreement with the infrared spectrum.

BIOLOGICAL RESULTS

The compounds appearing in Table I, with the exception of compound 9, were screened at the Parke Davis and Co. Laboratories for antibacterial activity. In addition, the screening program included bis-(4-aminophenyl)thiazolothiazole and thiazolothiazole-2,5-dicarboxylic acid and its dihydrazide and diisopropylidenehydrazide derivatives.

The compounds were tested against representative strains of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis*. None of the compounds showed antibacterial activity.

Compound 7 and thiazolothiazole-2,5-dicarboxylic acid were tested for antiviral activity with negative results.

Compounds 1, 2, 3, 4, and 14 showed no effect when tested for amebacidal activity.

Bis-(4-aminophenyl)-thiazolothiazole produced a 20% increase in blood cholesterol when administered to rats by intubation at a 5-mg. daily dose level for 7 days.

Compound 14, the 1,1-dimethyl-3-cyanopropyl derivative, was subjected to a preliminary pharmacological screening and appears to differ in its action from the usual central nervous system depressants.

The acute LD_{50} determined in mice is greater than 1250 mg./Kg. subcutaneously.

When given to dogs, rats, mice, and guinea pigs, orally or intramuscularly in doses of 2–200 mg./Kg., the compound induced normal sleep; that is, animals could be awakened by stimulation, but would subsequently return to sleep even in the laboratory environment. No excitement, respiratory depression, or general anesthesia was seen. Onset of action was 15–30 minutes; duration 2–3 hours. The electroencephalogram showed an ordinary sleep pattern during the action of the drug. The compound appeared qualitatively similar to trimeglamide (4) when directly compared.

The effect of sodium pentobarbital was intensified by the simultaneous administration of compound 14 whether measured by prolongation of sleeping time after anesthetic doses of pentobarbital or augmentation of the effect of subanesthetic doses. The tested compound was given in doses of 25–200 mg./Kg. intraperitoneally.

No autonomic or cardiovascular effects were demonstrable in anesthetized dogs with the smaller (50 mg./Kg.) doses tolerated by the unanesthetized animals.

No analgetic effect, strychnine antagonism, or protection against apomorphine emesis were demonstrable. Minor protection against pentylenetetrazol convulsions was shown.

The compound appears to have highly selective effects on the central nervous system, manifested almost purely by induction of sleep. However, it differs from the sedative-hypnotics in that an ex-

citement is not seen nor can anesthesia and medullary depression be induced by increasing the dose. Unlike the tranquilizers, this thiazolothiazole derivative does not alter autonomic function or exhibit evidences of central stimulation.

EXPERIMENTAL

General Procedures.—The molar ratio of reactants and other reaction data appear in Table I. *Procedure A.*—Following the published procedure (2), a mixture of dithio-oxamide and the aldehyde was heated under a stream of nitrogen in a wax bath. The reaction mixture was cooled and treated with ethanol. The product was filtered and recrystallized.

Procedure B.—Procedure A was modified by refluxing the reactants in dry pyridine for 60 hours. After cooling, the product was filtered and recrystallized.

Dihydrazide of Thiazolo[5,4-d]thiazole Dicarboxylic Acid.—A mixture of 0.5 Gm. (2 mmoles) of bis-(carbomethoxy)-thiazolothiazole, prepared from the corresponding acid (2) and diazomethane, 325 mg. (6 mmoles) of hydrazine hydrate (85% aqueous solution) and 125 ml. of ethanol was refluxed for 6 to 7 hours. The reaction mixture was concentrated by distillation under reduced pressure, filtered, and the product washed with ethanol. The yield was practically quantitative. A suitable recrystallization procedure was not achieved. The crude washed product, m.p. > 400°, gave the following analytical results.

Anal.—Calcd. for $C_6H_6N_6O_2S_2$: N, 32.56; S, 24.84. Found: N, 31.20; S, 24.78.

Diisopropylidenehydrazide of Thiazolo[5,4-d]thiazole Dicarboxylic Acid.—Five drops of glacial acetic acid was added to a suspension of 0.5 Gm. (2 mmoles) of the dihydrazide of thiazolothiazole dicarboxylic acid in 100 ml. of reagent acetone. After refluxing for 1 hour, the reaction mixture was concentrated under reduced pressure and filtered. The yield was practically quantitative. After recrystallization from acetone-ethanol, the product melted at 296–298°.

Anal.—Calcd. for $C_{12}H_{14}N_6O_2S_2$: N, 24.84; S, 18.95. Found: N, 25.28; S, 18.60.

Diamide of Thiazolo[5,4-d]thiazole Dicarboxylic Acid.—A fine suspension of bis-(carbomethoxy)-thiazolothiazole was prepared by dissolving 0.2 Gm. (0.78 mmole) of the ester in 5 ml. of boiling dioxane, followed by rapid cooling in an ice bath. Five milliliters of ammonium hydroxide (27%) was added and the mixture allowed to stand overnight at room temperature. After concentrating the mixture to one-half volume under reduced pressure, the white precipitate was filtered and washed with water and ethanol. The yield was practically quantitative. A suitable recrystallization procedure was not achieved. The crude, washed product did not melt when heated to 400°.

Anal.—Calcd. for $C_6H_4N_4O_2S_2$: N, 24.55; S, 28.00. Found: N, 24.30; S, 27.86.

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Pantothenyl Alcohol Effect on Delta-1-Cortisol-Induced Gastric Ulcers

By THOMAS A. LYNCH, WILBUR L. HIGHLEY, and ALBERT G. WORTON

The development of a standardized technique in assessing the ulcerogenic side effects of the corticosteroid drugs has provided an effective means of evaluating antiulcer agents. Approximately 90% incidence of gastric ulcers can be produced in fasting female rats by daily subcutaneous administration of 40 mg./Kg. delta-1-cortisol (D1C) for 4 days. Pantothenyl alcohol, extensively used in treating postoperative intestinal atony, was evaluated for antiulcer activity in such treated animals by two methods: (*a*) oral feeding during the 4 days induction period to determine effect on development on ulcers and (*b*) oral feeding after induction of ulcers to determine the effects on spontaneous healing. Pantothenyl alcohol did not reduce the degree of ulceration during the induction period, but markedly accelerated spontaneous ulcer healing.

THE IMPORTANCE of pantothenic acid to gastrointestinal integrity has been demonstrated by studies involving pantothenic acid deprivation and the pituitary axis. In 1943, Bly, *et al.* (1), demonstrated a 50% reduction of intestinal motility in pantothenic acid-deficient dogs. About the same time, Jurgens and Pfaltz (2) observed atony and distention of the gastrointestinal tract of pantothenic acid-deficient rats. These observations helped establish the basis for the use of pantothenic acid in the treatment of postoperative intestinal atony and gaseous intestinal distention (3–6). The discovery by Lipmann, *et al.* (7), that pantothenic acid formed an integral part of coenzyme A, and the pantothenic deficiency studies in humans by Bean,

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et al. (8), were important in establishing the link between pantothenic acid and intestinal formation of acetylcholine.

The relationship of pantothenic acid in resistance to stress and adrenocortical function has been presented by numerous workers (9–14). These studies implicate pantothenic acid as a protective factor against certain nonspecific stress effects.

Evidence for the correlation of the ulcerogenic effects of stress and adrenocortical activity has been demonstrated (15–21). However, Zucker (22) was the first to implicate pantothenic acid as a factor in ulcerogenesis. He demonstrated the formation of duodenal ulcers in rats on a pantothenic acid-deficient diet, which were healed by pantothenic acid administration. Evidence was presented in his study to show that the intact adrenal cortex and pituitary gland were necessary for development of these ulcers. An increase in sensitivity and vulnerability of the duodenal mucosa, as a target area, to the effects of adrenocortical hormone was suggested as a mode of ulcerogenesis.

Since there appeared to be good evidence that pantothenic acid deficiency was implicated in the etiology of experimental ulcers, it was decided to study the effect of pantothenyl alcohol in the prevention and treatment of such experimental ulcers in rats without a pantothenic acid depletion.

EXPERIMENTAL

The method of producing gastric ulcers was that of Robert and Nezamis (17), which consisted of administering daily subcutaneous doses of D1C for 4 days to fasted female rats. The animals were sacrificed with chloroform, the stomach removed, cut along the lesser curvature, and the mucosa grossly examined for incidence and severity of ulcers. A composite ulcer index was devised which measured the number, size, and per cent incidence of the ulcers.

Preventive Effects of Pantothenyl Alcohol in D1C-Induced Gastric Ulcers in Rats.—The method of producing ulcers in this study was essentially the same as described by the original workers (17). In the first part of this experiment, pantothenyl alcohol was administered during the ulcer induction period. Thirty female Wistar rats, weighing 167–236 Gm., were used. The animals were divided into three groups using five rats per cage. D1C suspended in a Tween base[1] was administered subcutaneously to two groups of 10 animals each, using a dosage of 1 ml. (40 mg.)/Kg. daily. Ten control rats were given an equivalent dosage of the vehicle by the subcutaneous route. Of the D1C-treated animals, 10 were given 1 mg./ml. of pantothenyl alcohol in their drinking water. All animals were sacrificed with chloroform after 4 days of steroid treatment and fasting, the stomachs removed, and the ulcer index determined. In addition, the adrenal glands were removed and weighed.

Pantothenyl Alcohol Treatment of D1C-Induced Gastric Ulcers in Rats.—Under similar conditions, the effect of pantothenyl alcohol was determined on the healing rate of gastric ulcers after the 4 days ulcer induction period. Thirty female Wistar rats, weighing 160–195 Gm., were used. All animals were given D1C subcutaneously under the same conditions as above. Following 4 days of ulcerogenic treatment, 25 mg./Kg. of pantothenyl alcohol was administered subcutaneously to 15 rats, which thereafter were given 1 mg./ml., of pantothenyl alcohol in their drinking water. Control animals were given an equivalent volume of normal saline subcutaneously. All animals were on Purina laboratory chow diet for the first day, followed by powdered whole milk[2] the remainder of the time. Animals were then sacrificed with chloroform at daily intervals for 3 days to determine the ulcer indexes. Thymus and adrenal gland weights were recorded throughout the experiment. Hemoglobin, erythrocyte, and leucocyte levels were determined on the third day of treatment.

An additional 52 rats (151–191 Gm.) were studied as above to confirm the 3 days pantothenyl alcohol treatment results and to extend the observations over a longer period. The procedure was the same as before, except that Purina laboratory chow was resumed as the diet on the ninth day, and pantothenyl alcohol was withdrawn from the rats on the thirteenth day of the 16 days treatment period. Hematologic tests were not made.

RESULTS

Preventive Effects of Pantothenyl Alcohol in D1C-Induced Gastric Ulcers in Rats.—When pantothenyl alcohol was given during the D1C gastric ulcer induction period, no improvement in the ulcer index was observed. Fasted rats, treated with D1C for 4 days, showed an ulcer index of 16.7, compared to 18.4 for rats on D1C and pantothenyl alcohol (Table I). The ulcer index, as determined by the method of Robert and Nezamis (17), is broken down for the individual rat to demonstrate the severity and incidence. The incidence of ulcers was 95% for the 20 D1C treated rats. These ulcers were found in the pyloric part of the stomach only. An ulcer index of 9.05 was also calculated for the control animals receiving the vehicle alone. Here the ulcers were found in the forestomach only. The results in Table I agree very favorably with those of Robert and Nezamis (17).

As shown in Table II, D1C reduced the adrenal gland weight by about 15%, but pantothenyl alcohol did not significantly influence this change. The mean body weight losses of the control, D1C and D1C-pantothenyl alcohol groups, during the 4 days fast period, were 25.4, 32.3, and 35.5%, respectively. The mean daily water consumption was about 100 ml./Kg. in all groups.

Pantothenyl Alcohol Treatment of D1C-Induced Gastric Ulcers in Rats.—Failure of pantothenyl alcohol administration to affect the development of D1C-induced gastric ulcers, prompted the in-

[1] Tween base, 5 mg. methylcellulose, 4 mg. Tween 80, 9 mg. sodium chloride, and 9 mg. of benzyl alcohol per 1 ml. of aqueous solution.

[2] M & R Dietetic Laboratories Inc., Columbus, Ohio.

TABLE I.—EFFECT OF PANTOTHENYL ALCOHOL ON D1C-INDUCED GASTRIC ULCERS IN FEMALE WISTAR RATS, 167–236 Gm.

Rat No.	Control: No. of Ulcers	Control: Severity	D1C[a]: No. of Ulcers	D1C[a]: Severity	D1C[a] and Pantothenyl Alcohol[b]: No. of Ulcers	D1C[a] and Pantothenyl Alcohol[b]: Severity
1	0	...	8	17	5	14
2	4	11	6	11	6	10
3	3	6	7	19	1	2
4	0	...	0	...	1	3
5	0	...	9	19	1	2
6	4	11	3	6	8	14
7	0	...	8	20	11	17
8	2	5	4	6	15	35
9	1	3	6	21	5	13
10	0	...	3	6	7	33
Ulcer index	9.05[c]		16.7[d]		18.4[d]	

[a] D1C suspension, 1 ml. (40 mg.)/Kg. subcutaneously per day for 4 days. [b] Pantothenyl alcohol, 1 mg./ml. in drinking water, equivalent to approximately 100 mg./Kg. [c] Ulcers found in forestomach only. [d] Ulcers found in pyloric part of stomach only.

TABLE II.—ADRENAL WEIGHTS OF FASTED FEMALE WISTAR RATS SUBJECTED FOR 4 DAYS TO PANTOTHENYL ALCOHOL AND THE STEROID ACTIONS OF D1C

Treatment	Mean Weight,[a] mg./100 Gm. ± S.E.
Control	45.1 ± 3.32
D1C[b]	38.3 ± 1.72
D1C[b] and pantothenyl alcohol[c]	37.5 ± 2.49

[a] Calculated from 10 rats per group. [b] D1C suspension 1 ml. (40 mg.)/Kg. subcutaneously per day for 4 days. [c] Pantothenyl alcohol, 1 mg./ml. in drinking water, equivalent to approximately 100 mg./Kg. per day for 4 days.

TABLE III.—EFFECT OF PANTOTHENYL ALCOHOL FOLLOWING THE DEVELOPMENT OF GASTRIC ULCERS BY D1C[a] IN FEMALE WISTAR RATS, 160–195 Gm.

Days of Treatment	Control: No. of Rats	Control: Ulcer Index	Pantothenyl Alcohol[b]: No. of Rats	Pantothenyl Alcohol[b]: Ulcer Index
0	2	20.1	...	...
1	2	20.3	3	20.7
2	7	17.0	6	11.5
3	5	17.4	5	9.4

[a] D1C suspension, 1 ml. (40 mg.)/Kg. subcutaneously per day for 4 days in fasted rats. [b] Pantothenyl alcohol, 25 mg./Kg. subcutaneously, given immediately after the 4 days ulcer induction period; thereafter, 1 mg./ml., equivalent to about 300 mg./Kg. daily, given in the drinking water.

TABLE IV.—EFFECT OF PANTOTHENYL ALCOHOL FOLLOWING THE DEVELOPMENT OF GASTRIC ULCERS BY D1C[a] IN FEMALE WISTAR RATS, 151–191 Gm.

Days of Treatment	Control: No. of Rats	Control: Ulcer Index	Pantothenyl Alcohol[b]: No. of Rats	Pantothenyl Alcohol[b]: Ulcer Index
0	2	26.8	...	...
1	2	28.2	3	22.5
2	7	24.0	6	20.8
3	5	24.3	5	18.1
5	5	20.5	5	13.9
9	3	17.2	3	12.1
16	3	8.1[c]	3	0.0

[a] D1C suspension, 1 ml. (40 mg.)/Kg. subcutaneously per day for 4 days in fasted rats. [b] Pantothenyl alcohol, 25 mg./Kg. subcutaneously, given immediately after the 4 days ulcer induction period; thereafter, 1 mg./ml., equivalent to about 300 mg./Kg. daily, given in the drinking water. [c] One rat with perforated ulcer.

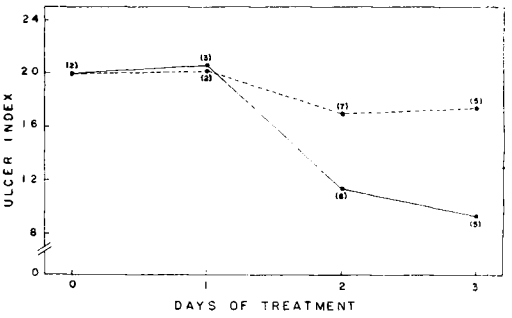


Fig. 1.—Effect of pantothenyl alcohol following the development of gastric ulcers in female Wistar rats. Ulcers induced by 40 mg./Kg. of D1C subcutaneously per day for 4 days in 30 fasted rats. Dotted line represents control animals. Unbroken line represents animals given 25 mg./Kg. of pantothenyl alcohol subcutaneously, immediately after the 4 days ulcer induction period, followed thereafter by 1 mg./ml. in the drinking water (equivalent to about 300 mg./Kg. daily). Number of animals in parentheses.

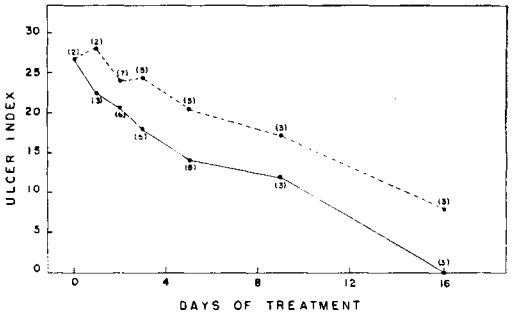


Fig. 2.—Effect of pantothenyl alcohol following the development of gastric ulcers in female Wistar rats. Ulcers induced by 40 mg./Kg. of D1C subcutaneously per day for 4 days in 52 fasted rats. Dotted line represents control animals. Unbroken line represents animals given 25 mg./Kg. of pantothenyl alcohol subcutaneously, immediately after the 4 days ulcer induction period, followed thereafter by 1 mg./ml. in the drinking water (equivalent to about 300 mg./Kg. daily). Number of animals in parentheses.

vestigation of pantothenyl alcohol effects on the spontaneous healing of ulcers. When pantothenyl alcohol was given on a treatment regimen to rats with D1C-induced gastric ulcers, definite declines below the control ulcer indexes were observed. Table III and Fig. 1 show a decrease of 32% in the ulcer index of pantothenyl alcohol-treated rats after 2 days of treatment and a 46% decrease after 3 days.

Hemoglobin, erythrocyte, and leukocyte levels on the third day of treatment were essentially the same in control and treated groups. Determination of thymus and adrenal gland weights failed to reveal a definite pattern.

Water consumption during the 4 days D1C treatment period was approximately 50% greater than before, but increased about twofold in both groups when food and treatment were instituted. Body weight, reduced about 33%, remained at this level in both groups even though the animals were

receiving food during the treatment period. Unabsorbed D1C remained in the subcutaneous site in all of the animals after 3 days of treatment and may have accounted for the absence of weight gain.

The results of the additional 52 rats tabulated in Table IV and depicted in Fig. 2, show an initially higher ulcer index than before, but essentially the same reduction of ulcer index by pantothenyl alcohol during the first 3 days of treatment. The ulcer indexes of the pantothenyl alcohol-treated rats were consistently lower than the controls and averaged about 23% below controls during the first 9 days of treatment. On the sixteenth day, no ulcers were found in the pantothenyl alcohol group, while one perforated ulcer was found in the control group. Extensive multiple erythematous eruptions of the forestomach occurred in both treated and control groups on the fifth day of treatment only. These were more severe in the control rats. No significant changes in thymus and adrenal gland weight patterns were noted.

The relative water consumption and weight change pattern during the first 3 days of treatment was essentially the same as noted earlier. Water consumption continued at about the same rate until the end of the experiment. After the fifth day of treatment, control rats began to gain weight, concomitant with the disappearance of D1C deposits in the subcutaneous tissues; however, on the ninth day, pantothenyl alcohol-treated animals showed obvious signs of alopecia and became emaciated, even though their ulcer index was declining more rapidly. When pantothenyl alcohol was withdrawn from the drinking water on the thirteenth day of treatment, the animals rapidly regained 10% of their body weight in 2 days to match that of the controls, and signs of emaciation disappeared.

DISCUSSION

The production of gastric ulcers in fasting rats by use of D1C confirms the work of Robert and Nezamis (17), and illustrates the efficacy of the method.

The failure of pantothenyl alcohol to prevent D1C-induced gastric ulcers, in contrast to its apparent accelerating effect on healing, may be due to the overwhelming action of the massive doses of steroid during the ulcer induction period.

The sustained depression of body weight, following the 4 days ulcer induction period, corresponds to the presence of D1C deposits in the subcutaneous tissue, and probably prevented a more rapid spontaneous ulcer healing.

The mechanism of pantothenyl alcohol in accelerating the healing of experimental gastric ulcers is obscure. Zucker (22) has suggested as the etiology of the experimental rat duodenal ulcer during pantothenic acid deficiency, that the target area or duodenal mucosa becomes sensitized to the ulcerogenic effect of the adrenocortical steroids. In D1C-induced gastric ulcers, existing levels of mucosal pantothenate may be less adequate in maintaining and restoring cellular integrity. Pantothenyl alcohol may aid in the maintenance and reparative processes of the gastric mucosa by providing a greater level of tissue pantothenate.

The emaciation and the alopecia observed in the rats on the ninth day of treatment is unusual in that the effects appeared as a result of the pantothenyl alcohol treatment. Unna and Greslin (23) found no toxicity when 200 mg. of calcium pantothenate was fed daily to rats for a period of over 6 months. Their average dosage was roughly three times that of pantothenyl alcohol used in the present experiment. The observed toxic effects further emphasize the role of pantothenic acid under stress conditions.

SUMMARY

1. Gastric ulcers were experimentally produced in fasted female rats by subcutaneous administration of D1C for 4 days.

2. Pantothenyl alcohol given during the induction period did not significantly alter the development of D1C-induced gastric ulcers.

3. Pantothenyl alcohol markedly accelerated the healing of gastric ulcers when given during the recovery period. Complete healing was observed by 16 days in the pantothenyl alcohol group, but required a longer period in the control animals.

4. Pantothenyl alcohol-treated animals showed obvious evidence of toxicity, characterized by emaciation and alopecia on the ninth day of treatment, which disappeared upon withdrawal of the drug.

5. The possible role of pantothenyl alcohol, relative to its effect on D1C-induced gastric ulcers, is discussed.

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Quantitative Study of Chelates of Pyridine-2-aldoxime

By SANFORD BOLTON and ROBERT I. ELLIN†

Acid dissociation constants of pyridine-2-aldoxime and its affinity for various metal ions were determined by means of potentiometric titrations. Stable chelates were formed with the following metal ions, listed in order of stability: ferric > cupric > cobaltous > ferrous > nickelous > mercuric > zinc > cadmium >manganous. Calcium and magnesium ions showed no evidence of chelate formation. The unusually high stabilities of the cobaltous and ferrous chelates suggest that the oxime exhibits a specific affinity for these ions. All of the 1:1 chelates showed a strong tendency to hydrolyze, as indicated by results obtained from titrations of solutions containing the reactants in a 1:1 ratio. Colored species were formed in the ferrous, ferric, cobaltous, nickelous, and cupric systems. In particular, the ferrous chelate formed an intense red solution, suggesting possible application of the oxime as an analytical reagent for ferrous ion.

THE CHELATING ability of the oxime group has been recognized for some time; however, relatively little work on the stability of oxime chelates has been reported in the literature. A notable exception is dimethylglyoxime which, because of its analytical applications, has been the subject of extensive investigations (1–3). Recently a few papers have appeared which were particularly concerned with oxime chelation. Murmann has calculated stability constants of chelates of 2-methyl-2-amino-3-butanone oxime and some of its derivatives (4). Proposed chelate structures demonstrated formation of hydrogen bonded species, similar to those observed for dimethylglyoxime chelates. Banerjea and Tripathi noted the intense colors of cuprous and ferrous chelates of methyl-2-pyridyl ketoxime and described the use of this oxime as an analytical reagent for these metals (5). During the course of the present investigation, a paper by Krause and Busch was published, which described in detail the isolation of and formulas for nickelous, palladium, and platinum chelates of pyridine-2-aldoxime (6).

The primary purpose of this investigation was to study the ability of pyridine-2-aldoxime to form chelates with selected metal ions. These metal complexes may be of value in the treatment of poisoning by organophosphate anticholinesterases. Wagner-Jauregg and associates (7) demonstrated that the hydrolysis rate of diisopropylphosphorofluoridate (DFP) is greatly accelerated in the presence of various cupric chelates; the α,α'-dipyridyl chelate, which is related structurally to pyridine-2-aldoxime, showed the strongest catalytic activity. A similar study by Courtney and associates (8) described a number of metal chelate compounds which acted as catalysts in hydrolyzing DFP and isopropyl methylphosphonofluoridate (sarin). Although these complexes are efficient for decomposing organophosphate anticholinesterases, only a few appear to have been tested *in vivo* (9).

Received April 28, 1961, from The College of Pharmacy, University of Rhode Island, Kingston.
Accepted for publication June 30, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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EXPERIMENTAL PROCEDURE

Reagents.—Pyridine-2-aldoxime, recrystallized from 50% ethanol, m.p. 113°; potassium nitrate, analytical reagent; sodium hydroxide solution (carbonate free) and hydrochloric acid, both standardized by conventional methods; ferric nitrate ferrous chloride, cobaltous nitrate, nickelous nitrate, cupric nitrate, mercuric chloride, cadmium nitrate, zinc nitrate, manganous nitrate, calcium chloride, and magnesium nitrate, all analytical reagent grade. Purities of the metal salts, with the exception of ferrous chloride, were checked by titrating with an 0.05 *M* solution of the disodium salt of EDTA, analytical reagent, according to methods suggested by Chaberek and Martell (10), using Eriochrome-schwartz T and murexide as indicators. Ferrous chloride was analyzed by a conventional method.

Procedure.—The experimental method consisted of potentiometric titrations of the oxime alone and in the presence of various metal ions. The ratio of ligand to metal salt in solution was varied to determine both the qualitative and quantitative nature of the interaction (11). The ionic strength for all solutions was maintained at 0.1 *M* by the addition of potassium nitrate. To insure the exclusion of carbon dioxide, nitrogen was passed through the solutions during the course of the titrations. During each titration, enough time was allowed between addition of increments of base to make certain that equilibrium was attained. This precaution was especially critical in the cobaltous and ferrous systems, where in many instances a considerable period of time elapsed before the pH readings became constant. All measurements were made at 24 ± 0.5° with a Beckman model G pH meter, equipped with extension electrodes. Spectrophotometric measurements were made with a Bausch and Lomb Spectronic 20 colorimeter.

THEORY

Chelate Formation.—Chelates of pyridine-2-aldoxime may take either of the two forms illustrated in Fig. 1. If equilibrium 2 is predominant it is possible that the resulting chelate may act as a relatively weak acid

$$C_5H_4N{-}CH{=}NOH + M \rightleftarrows [(C_5H_4N{-}CH{=}NOH)M] \rightleftarrows (C_5H_4N{-}CH{=}NO^-)M + H^+$$

However, analysis of titration data on the basis of complete dissociation of the aldoxime hydrogen yielded constant values of chelate stability constants. This observation suggests that the intermediate, undissociated form represented above (bracketed) is either absent or present in only negligible amounts in these systems. Therefore, it is impossible to distinguish between the two equilibria represented in Fig. 1 by means of potentiometric titrations.

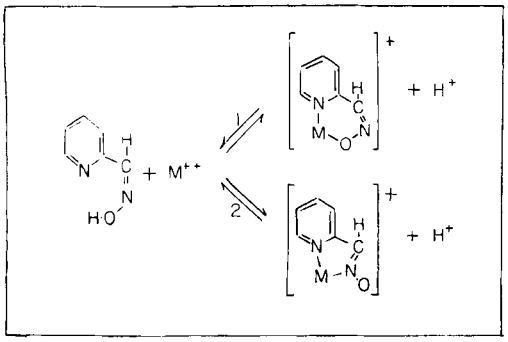


Fig. 1.—Chelates of pyridine-2-aldoxime.

Acid Dissociation Constants.—A method suggested by Chaberek and Martell (12) was used to determine the acid dissociation constants of pyridine-2-aldoxime (pa) from titration data (Fig. 2). The relevant constants are

$$K_{a1} = (HA)(H^+)/(H_2A^+) = 2.05 \times 10^{-4} \quad \text{(Eq. 1)}$$

$$K_{a2} = (A^-)(H^+)/(HA) = 9.50 \times 10^{-11} \quad \text{(Eq. 2)}$$

where $(H_2A^+) = C_5H_4NH^+{-}CH{=}NOH$, $(HA) = C_5H_4N{-}CH{=}NOH$, and $(A^-) = C_5H_4N{-}CH{=}NO^-$. The first dissociation constant (K_{a1}) is of significance in solutions of pH less than 6. When calculating chelate stability constants at higher pH's this equilibrium may be neglected.

Chelate Stability Constants.—The applicable equilibria involved in chelate formation may be expressed as follows

$$M^n + A^- \rightleftarrows MA^{n-1} \qquad K_{MA} = (MA^{n-1})/(M^n)(A^-) \quad \text{(Eq. 3)}$$

$$MA^{n-1} + A^- \rightleftarrows MA_2^{n-2} \qquad K_{MA2} = (MA_2^{n-2})/(MA^{n-1})(A^-) \quad \text{(Eq. 4)}$$

$$MA_2^{n-2} + A^- \rightleftarrows MA_3^{n-3} \qquad K_{MA3} = (MA_3^{n-3})/(MA_2^{n-2})(A^-) \quad \text{(Eq. 5)}$$

where M^n = metal ion of charge n and A^- is defined above. The stability constants were calculated from potentiometric data by an adaptation of Bjerrum's method (13). Derivations of the equations involved have been reported elsewhere (12).

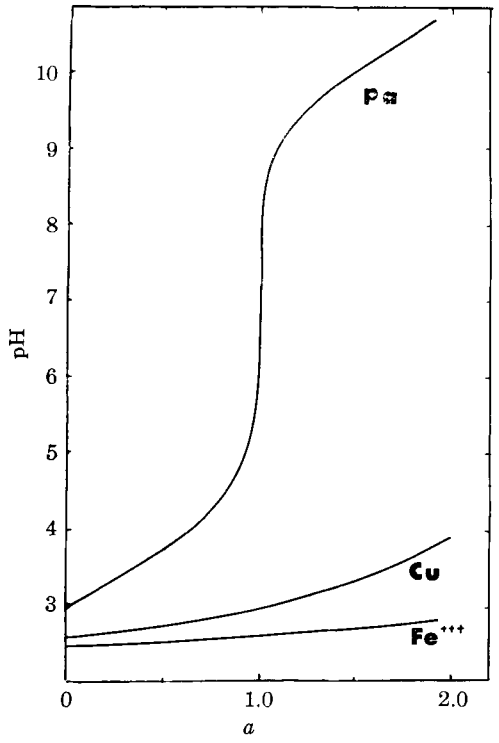


Fig. 2.—Titration curves of pyridine-2-aldoxime (pa) plus one equivalent of HCl. $C_{pa} = 0.008$ *M*. $C_{pa}/C_M = 0.004/0.004$ *M* = 1. *a*, Moles of base per mole of metal ion.

The value of (A^-) may be determined from titration data as follows

$$(A^-) = \frac{(C_A) - a(C_A) - (H^+) + (OH^-)}{\frac{(H^+)}{K_{a2}} + \frac{2(H^+)^2}{K_{a1}K_{a2}}} \quad \text{(Eq 6)}$$

where C_A = total molar concentration of ligand in the system and a = moles of base added per mole of ligand.

The number of moles of ligand bound per mole of metal ion, $\bar{n}$, has been called the "formation function" by Bjerrum (13).

$$\bar{n} = \frac{(C_A) - (A_{free})}{(C_M)} = \frac{(C_A) - (A^-)[1 + (H^+)/K_{a2} + (H^+)^2/K_{a1}K_{a2}]}{(C_M)} \quad \text{(Eq. 7)}$$

where (A_{free}) = total concentration of all free ligand species and (C_M) = total concentration of metal ion in the system; $\bar{n}$ may be calculated from Eqs. 6 and 7.

Bjerrum has shown that log K_{MA}, log K_{MA2}, and log K_{MA3} may be determined from a plot of $\bar{n}$ *vs.* pA, where pA = $-\log (A^-)$, if the successive K values are sufficiently different

$$\log K_{MAn} = p\text{A}, \text{ when } \bar{n} = n - 0.5 \quad \text{(Eq. 8)}$$

$$\log (K_{MAn}K_{MAn-1}) = 2p\text{A}, \text{ when } \bar{n} = n - 1 \quad \text{(Eq. 9)}$$

If the K values, as obtained by this method, do not

differ greatly in magnitude, a method of successive approximations may be used to determine more exact values of the stability constants (13). In the present investigation, K_{MA} and K_{MA2} for the mercuric, zinc, and cadmium chelates were determined by the latter method.

Spectrophotometric measurements may be employed to determine stability constants if the absorption of all pertinent species is known. In the present work, K_{MA} for the ferric chelate was determined by this method. The extinction coefficient for the 1:1 chelate was determined by measuring the absorption of a solution containing ferric ion and oxime, in which the ferric ion was present in large excess to insure complete formation of the 1:1 chelate. The concentration of chelate formed in solutions where chelation was not complete could then be determined from spectrophotometric measurements and K_{MA} calculated according to the following relationship

$$K_{MA} = [(x)/(C_M - x)(C_A - x)] \times [(1 + (H^+)/K_{a2} + (H^+)^2/K_{a1}K_{a2}] \quad \text{(Eq. 10)}$$

where x = molar concentration of chelate.

RESULTS AND DISCUSSION

Titration Curves.—The formulas and stability constants of the metal chelates were determined from potentiometric titration curves illustrated in Figs. 2–7. Although manganous ion showed evidence of a weak interaction, precipitation occurred early in the titration, and stability constants could not be calculated. Calcium and magnesium ions showed no evidence of chelate formation. Therefore, the results for the manganous, calcium, and magnesium ions have been omitted from the data.

Figure 2 shows the titration curves for pyridine-2-aldoxime alone and in the presence of equimolar concentrations of ferric and cupric ions. One equivalent of hydrochloric acid was added to the titration mixtures in order to determine the first dissociation constant for the oxime (K_{a1}). Since more than half of the oxime was bound to both ferric and cupric ions before the addition of the acid, the titrations illustrated in Fig. 2 were necessary to determine values of (A^-) at lower values of $\bar{n}$.

Titration curves for a 1:1 ratio of reactants are shown in Fig. 3. Precipitation is indicated by the letter x. In all cases where precipitation did not occur before the completion of the titration, a definite inflection was observed at some point after the addition of one equivalent of base. Such results indicate hydrolysis and/or polymerization of the chelate species (14). In the cases of the cadmium, zinc, and nickelous ions, the end point observed at two equivalents of base suggests one of the following reactions

$$H_2O\text{—}MA^+ + OH^- \rightleftharpoons HO\text{—}MA + H_2O$$

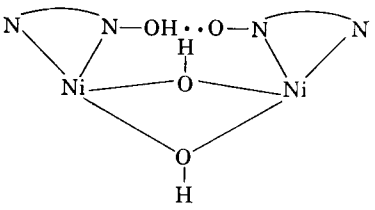
$$2H_2O\text{—}MA^+ + 2OH^- \rightleftharpoons AM\begin{matrix}O\text{—}H\\ \diagup\ \diagdown\\ O\text{—}H\end{matrix}MA + 2H_2O$$

The titration curve for the nickelous ion is also characterized by the appearance of a break at one and one-half equivalents of base. Possible explanations of this phenomenon are: (*a*) dimerization is occurring stepwise

$$2H_2O\text{—}MA \underset{}{\overset{OH^-}{\rightleftharpoons}} \left[\begin{matrix}H\\ |\\ AM\text{—}O\text{—}MA\\ |\\ H_2O\end{matrix}\right]^+ + H_2O$$

$$\overset{OH^-}{\rightleftharpoons} AM\begin{matrix}O\text{—}H\\ \diagup\ \diagdown\\ O\text{—}H\end{matrix}MA + H_2O$$

and (*b*) a stable, dimeric, hydrogen-bonded species is formed



The breaks at approximately one and one-quarter and one and two-thirds equivalents of base for copper are very difficult to rationalize. It would appear that formation of a complex polymeric species is occurring in these solutions. Titration of the ferric chelate was complete upon addition of three equivalents of base, and this fact suggested that a stable dihydroxo-ferric chelate was formed.

When the reactants were present in a molar ratio of two aldoximes to one metal ion, an end point was observed at two equivalents of base (Fig. 4). These

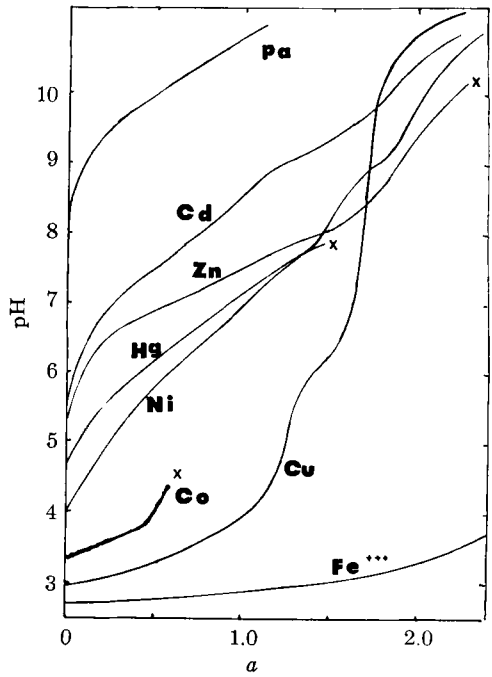


Fig. 3.—Titration curves of pyridine-2-aldoxime (pa). $C_{pa} = C_M = 0.004$ *M*. *a*, Moles of base per mole of metal ion; *x*, precipitation.

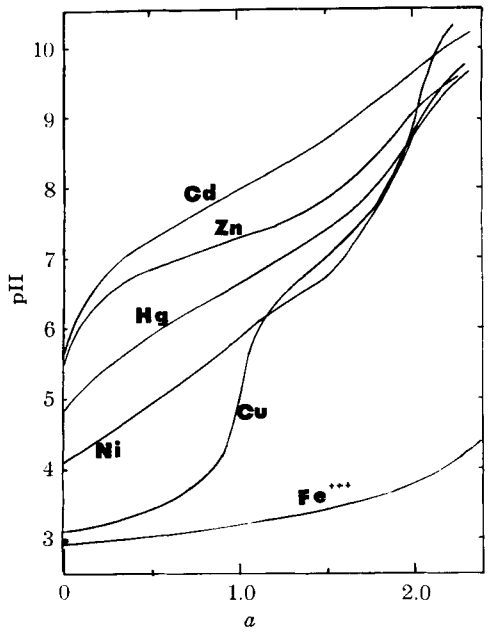


Fig. 4.—Titration curves of pyridine-2-aldoxime. C_{pa} = 0.004 *M*. C_{pa}/C_M = 2. *a*, Moles of base per mole of metal ion.

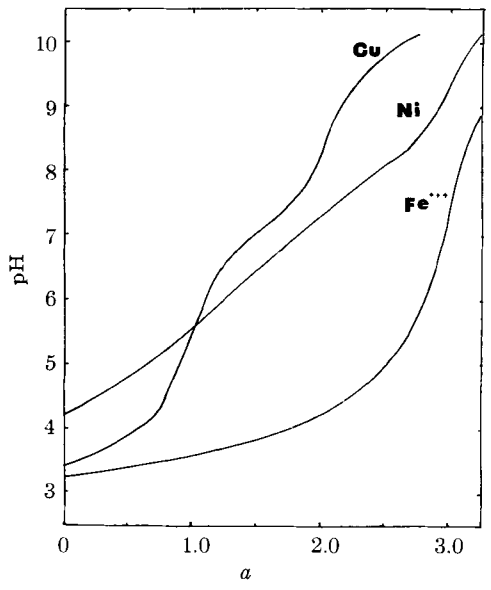


Fig. 5.—Titration curves of pyridine-2-aldoxime. C_{pa} = 0.004 *M*. C_{pa}/C_M = 3. *a*, Moles of base per mole of metal ion.

curves are indicative of the formation of stable 2:1 chelates. The ferric ion again showed a break at three equivalents of base, an indication of formation of a hydroxo-2:1 chelate. The sharp breaks at one and two equivalents of base for the copper chelate is indicative of a large "spreading factor" (13) between the first and second chelate stability constants.

Figure 5 illustrates titration of the nickelous,

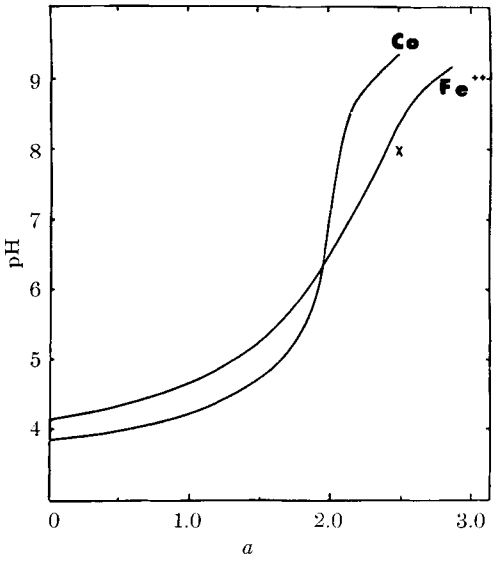


Fig. 6.—Titration curves of pyridine-2-aldoxime. C_{pa} = 0.0032 *M*. C_M = 0.00048 *M*. *a*, Moles of base per mole of metal ion; *x*, precipitation.

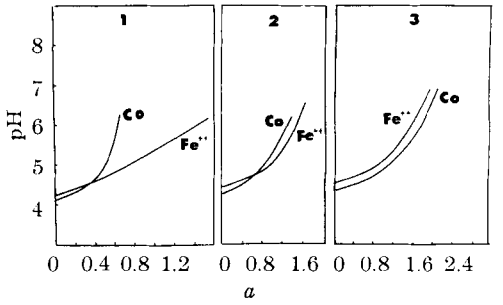


Fig. 7.—Titration curves of pyridine-2-aldoxime. 1, C_{pa} = C_M = 0.00032 *M*. 2, C_{pa} = 0.00032 *M*. C_{pa}/C_M = 2. 3, C_{pa} = 0.00032 *M*. C_{pa}/C_M = 3. *a*, Moles of base per mole of metal ion.

cupric, and ferric systems, containing the reactants in a ratio of three ligand to one metal ion. The single break at three equivalents for the nickelous and ferric ions indicates formation of 3:1 chelates. The curve for the cupric ion shows that stable chelates of an order higher than 2:1 are not being formed. Because zinc, cadmium, and mercuric ions showed no evidence of forming 3:1 chelates under similar conditions, the results are not shown here.

The ferrous and cobaltous interactions (Figs. 6 and 7) have been treated separately for several reasons. Precipitation of a chelate species occurred before completion of the titration in solutions comparable in strength to those illustrated in Figs. 3–5. Further, equilibrium occurred very slowly and it was extremely difficult to determine the point at which the pH of these solutions remained steady. To overcome these difficulties, studies were made at a larger ratio of ligand to metal ion (Fig. 6). These results show the formation of a 2:1 cobaltous and a 3:1 ferrous chelate. In the ferrous system, the

fact that after the addition of two equivalents of base the buffer region occurred at lower pH values was indicative of formation of a 3:1 species. An end point at three equivalents of base was not observed because precipitation occurred before this point was reached. Although equilibrium occurred rather quickly in these systems, cobaltous chelation was virtually complete before the addition of base and stability constants could not be determined. Therefore, studies were made at lower concentrations of reactants (Fig. 7). Again, precipitation occurred in some cases and the equilibrium pH could not be determined at elevated pH readings (pH 5–6) but enough values were obtained at lower pH values to yield reproducible stability constants.

Analysis of Colored Species.—The above discussion does not take into account the possibility that the end points observed in the 2:1 and 3:1 systems could also be due to titration of hydroxo-chelates. The method of Job (15) was used to demonstrate that 2:1 and 3:1 chelates were indeed forming in these systems. This method was applicable to those chelates which formed colored solutions (Table I.)

TABLE I.—COLORS OF CHELATE SOLUTIONS

Chelate	Color		
	Low pH		High pH
Ferric	Deep orange		Brown-red
Cupric	Light green		Deep green
Cobaltous	Yellow		Yellow orange
Ferrous	Red		Deep red
Nickelous	Yellow		Orange
Mercuric		Colorless	
Cadmium		Colorless	
Zinc		Colorless	

Among these solutions only the ferrous chelate showed an absorbance peak in the visible spectrum (515 mμ). [The ferrous chelate formed an intense red solution under alkaline conditions. In dilute, alkaline (pH 10–11) solutions of ferrous ion containing excess oxime, no precipitation occurred and the color was stable for approximately 12 hours. These facts suggest possible use of the oxime as an analytical reagent for ferrous ion.] For the other solutions, a convenient wavelength was selected for analysis as indicated in Fig. 8. Although the data are not included here, the nickelous ion showed evidence of 2:1 chelate formation at high pH values, according to the method of Job. This fact seems to suggest that under these conditions the 3:1 species dissociates to the 2:1 chelate. Otherwise, the results shown in Fig. 8 are completely compatible with the previous discussion.

Isolation of Chelate Species.—A mixture of 0.02 *M* mercuric chloride and 0.02 *M* pyridine-2-aldoxime yielded a white, crystalline precipitate.

Anal.—Calcd. for $(C_5H_4N{-}CH{=}NOH)HgCl_2$: N, 7.15; O, 4.07; Hg, 51.0, Cl, 18.0. Found: N, 7.3; O, 4.2; Hg, 50.4; Cl, 17.8.

It is interesting to note that this insoluble species probably contains the oxime in an undissociated form, since insoluble chelates usually have a zero net charge. However, as mentioned previously, this undissociated form is probably present in negligible amounts in more dilute solutions in which the chelate is soluble.

Attempts to isolate pure chelates formed by cobaltous and ferrous ions failed because the insoluble species precipitated in a colloidal form. Extreme conditions of pH and temperature applied to more concentrated solutions (0.02 *M*) of these chelates resulted in appreciable precipitation. However, analysis showed that these precipitates probably were either mixtures of chelate and metal hydroxides or complicated polymeric forms. Analysis of precipitates obtained by adding excess base to 0.02 *M* solutions of the zinc and cupric chelates also showed no correspondence to a single, simple species. No insoluble chelates could be isolated from the other systems.

Formation Functions.—The formation functions illustrated in Fig. 9, were calculated according to Eq. 7 from data derived from Figs. 2–7. This plot clearly shows the relationship of the stability constants of the various chelates. With one exception, $\bar{n}$ *vs.* *p*A values calculated from titration curves of different ligand:metal ratios were in good agreement. In the nickelous system, results obtained from the 1:1 curve, which described K_{MA}, were different from corresponding results derived from the 2:1 and 3:1 curves. Since the 2:1 and 3:1 titration curves yielded data which were in good agreement, the nickelous chelate formation curve in Fig. 9 was constructed from these data.

The large breaks for the ferrous chelate at $\bar{n}$ = 2 and for the cupric chelate at $\bar{n}$ = 1 indicate a large value for the "spreading factor" between successive stability constants. The other curves show no such inflections, indicating relatively small differences between successive stability constants.

Stability Constants.—Table II lists the Stability constants determined in this study. Previously reported values for dimethylglyoxime are included for purposes of comparison. The constants were determined from the formation functions by methods previously described. Values of $\bar{n}$ greater than 2.3 could not be determined for the ferrous system. Therefore, the value of log K_{MA2} reported in Table II is an approximation, determined from the value of (A^-) at $\bar{n}$ = 2 (Eq. 9). K_{MA} for the ferric chelate was also determined at pH 2 by a spectrophotometric method outlined in the calculations section (Eq. 10) and was in good agreement with the value determined potentiometrically (Table III). The fact that formation of a 1:1 chelate is the predominant equilibrium in solutions of pH 2 was demonstrated by Job's method (Fig. 8). This check was

TABLE II.—STABILITY CONSTANTS

Metal Ion	Pyridine-2-aldoxime			Dimethylglyoxime[a,b]	
	Log K_{MA}	Log K_{MA2}	Log K_{MA3}	Log K_{MA}	Log K_{MA2}
Ferric	11.4	10.3	8.4	..	..
Cupric	10.8	6.0	..	11.9	11.4
Cobaltous	9.6	8.7	..	9.8	9.1
Ferrous	9.4	8.0	5.1[c]	..	..
Nickelous	8.1[d]	6.1	5.0	11.2	10.5
Mercuric	6.5	5.7	..	..	..
Zinc	5.5	5.3	..	7.7	6.2
Cadmium	5.2	4.4	..	5.7	5.0

[a] In 50% dioxane. [b] From Bjerrum, J., *et al.*, "Stability Constants," Part 1: Organic Ligands, The Chemical Society, London, 1957. [c] Estimated value. [d] Determined from 2:1 and 3:1 titration curves.

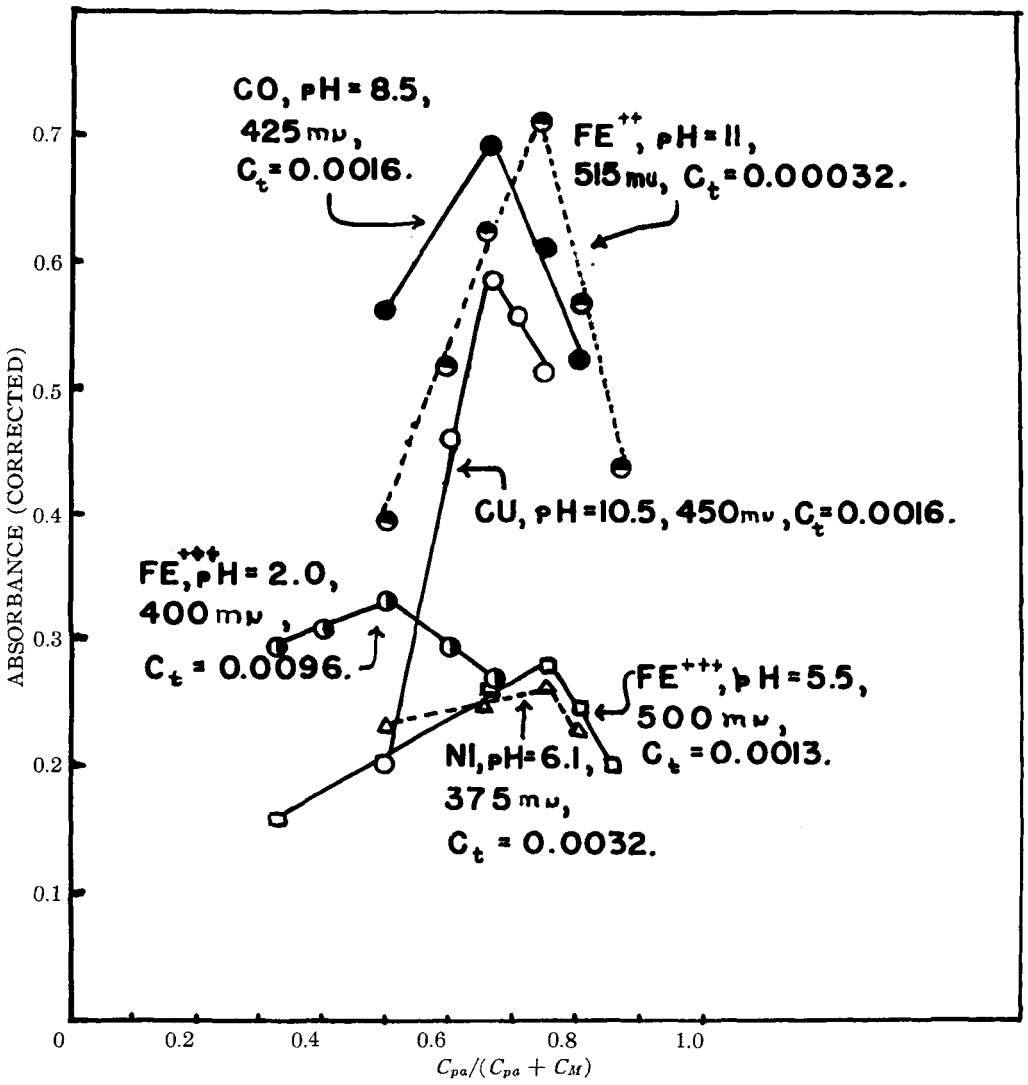


Fig. 8.—Composition curves (method of Job). $C_t = C_{pa} + C_M$.

TABLE III.—STABILITY CONSTANT OF 1:1 FERRIC CHELATE DETERMINED SPECTROPHOTOMETRICALLY[a]

$(C_A)/[(C_{pa}) + (Fe^{+++})]$	Absorbance, 400 mμ (Corrected)	Log K_{MA}
0.33	0.295	11.27
0.40	0.306	11.23
0.50	0.326	11.25
0.60	0.291	11.21
0.67	0.264	11.20

[a] pH = 2. Molar absorbance of chelate (400 mμ) = 556. $(Fe^{+++}) + (C_{pa}) = 0.0096$ *M*.

made because large errors may be introduced in the determination of a very large stability constant when a relatively small amount of (A^-) is in equilibrium with the chelate.

As previously noted, analysis of the 1:1 titration curve for the nickelous chelate resulted in values of K_{MA} which differed from values obtained from the 2:1 and 3:1 curves. Algebraic calculation of log K_{MA} (12) from 1:1 data at low values of $\bar{n}$ (where chelate hydrolysis does not interfere with the calculations) yielded values which were not constant, varying from approximately 7.5 to 7.0 at $\bar{n}$ equal to 0.21 and 0.36, respectively. These low, inconsistent results are difficult to explain; but they may possibly be due to incomplete dissociation of the oxime hydrogen from the chelate in these solutions. For example, the formation of a dimeric species of a structure similar to that previously suggested (see under discussion of the 1:1 titration curve of the nickelous chelate) could account for the inconsistency of these calculations.

The relative stabilities of transition metal chelates have been correlated with the atomic number of the metal ion (16). This relationship is shown graphically in Fig. 10 for chelates of EDTA, ethylenediamine, and pyridine-2-aldoxime; for which the maximum value of the stability constant occurs for the copper system. A further increase of the

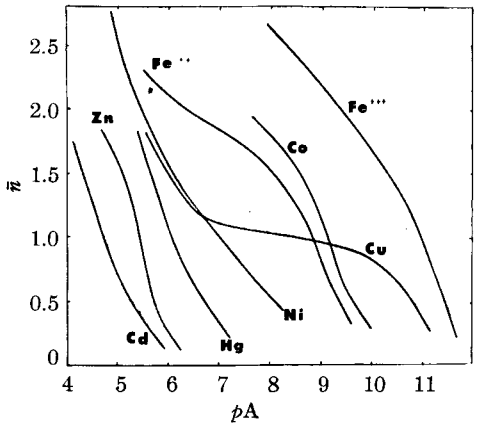


Fig. 9.—Formation functions. pA = log [1/(A$^-$)]. $\bar{n}$ = (Ligand$_{bound}$)/(C_M).

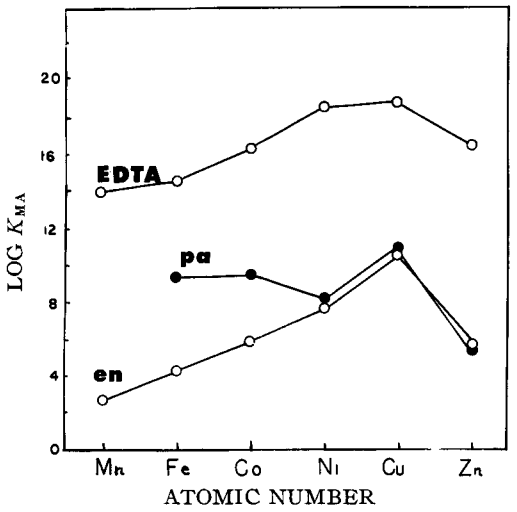


Fig. 10.—Correlation of log K_{MA} with atomic numbers of transition metals (and zinc). en, Ethylenediamine; pa, pyridine-2-aldoxime.

atomic number by one, going to zinc, results in a decrease of the stability constant. It should be noted that the ferrous and cobaltous chelates of pyridine-2-aldoxime show large deviations from the usual results; the affinity of the oxime for these ions is greater than that which would be expected from results observed in other chelate systems. It has been previously noted that complexation may occur through either the oxime nitrogen or oxygen (Fig. 1). The actual configuration may depend on particular circumstances such as pH and the properties of the metal ion. The "specificity" observed for the ferrous and cobaltous chelates may be due to an optimum set of such conditions for the formation of a chelate ring.

SUMMARY

1. The acid dissociation constants of pyridine-2-aldoxime and its chelate stability constants with various metal ions have been determined by means of potentiometric titrations. The stabilities of the 1:1 chelates were in the following order: $Fe^{+++} > Cu^{++} > Co^{++} > Fe^{++} > Ni^{++} > Hg^{++} > Zn^{++} > Cd^{++} > Mn^{++}$. Ferrous and cobaltous ions appeared to have unusually high affinities for the oxime. Calcium and magnesium ions showed no evidence of complexations. Cu^{++}, Co^{++}, Hg^{++}, Zn^{++}, and Cd^{++} ions formed stable 2:1 complexes, while Fe^{+++}, Fe^{++}, and Ni^{++} ions formed 3:1 species.

2. One to one titration curves indicated that hydrolysis and/or polymerization of the 1:1 chelates was occurring at elevated pH values.

3. Colored species were formed in the Fe^{+++}, Cu^{++}, Co^{++}, Ni^{++}, and Fe^{++} systems. In particular, the ferrous chelate formed an intense stable red solution at high pH values.

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Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters III

Biological Activities in Fruit Flies

By FLORENCE C. KLEE† and ERNST R. KIRCH

Biological responses of the flies which eventually did not survive suggest that the compounds are cholinesterase inhibitors. Male fruit flies are the most susceptible in 0.01 and 0.001% w/v solutions. Concentration-toxicity and structure-toxicity relationships are discussed.

THE SYNTHESIS of thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters (1) and their biological activities in guppies (2) have been published recently.

The purpose of this investigation was to observe the biological responses of fruit flies (*Drosophila melanogaster*) exposed to 0.1, 0.01, and 0.001% w/v aqueous solutions or suspensions of thiophosphoric acid derivatives which have structures similar to those of known acetylcholinesterase inhibitors. The derivatives have the general formula $-\overset{|}{N}-\overset{S}{\overset{\|}{P}}(X)_2$ where N represents the amine or amino acid ester moiety and $(X)_2$ = $(O\text{-alkyl})_2$, $(O\text{-}C_6H_5)_2$, $(C_2H_5O)Cl$, or $(Cl)_2$.

If these derivatives are anticholinesterases certain biological responses should be observed such as secretion of saliva, dizziness, evacuation of rectum, convulsions, and total paralysis (death).

EXPERIMENTAL

A cornmeal-molasses agar medium (3) was prepared, poured into Erlenmeyer flasks, and autoclaved after cotton stoppers were inserted. Several pairs of red eye and white eye female and male flies were introduced into each flask, and within 11 days many flies developed. One or two-day old flies were used in the investigation of the insecticidal activity of the compounds.

Aqueous suspensions or solutions (0.1, 0.01, and 0.001% w/v) of each compound listed in Table I were tested with 100 flies consisting of 50 males and 50 females in different test tubes. By separating the sexes the biological responses of each sex could be easily observed.

A 9-cm. filter paper was inserted into a 6-inch Pyrex test tube and firmly pressed against the glass surface about one inch from the top of the tube. Young flies were anesthetized with ether and transferred to the paper-lined tubes. Cotton stoppers were inserted and the flies were allowed to recover from the ether. The solution to be tested (0.9 ml.) was slowly introduced onto the filter paper by means of a syringe supplied with a long needle. The flies were periodically observed and the results at the end of 24 hours are presented in Table I. When distilled water was used instead of a suspension or solution of a compound under test it was observed for control purposes that both sexes survived after 24 hours.

Inhibition of cholinesterase is suggested from observations of the fruit flies which eventually did not survive in test tubes containing aqueous suspensions or solutions of the compounds. The flies were extremely agitated and frequently exhibited muscle contractions (of the proboscis and leg) and wing flutter (convulsions). Later they fell on their dorsal side and remained in this position for many hours due to dizziness and leg unsteadiness. All attempts to place them on their feet were unsuccessful.

Sex Differences.—Neither sex survived in 0.1% w/v aqueous suspensions or solutions of 10 of the 17 compounds in Table I.

Ethylamine Series.—Three of the esters which are extremely toxic to male fruit flies are not as toxic to the female flies. About 37% of the females survived after being in contact for 24 hours with 0.1% suspensions of the dichloro derivative or the tertiary ester, and 74% survived after being subjected to the isobutyl ester. The phenyl ester is somewhat toxic (32%) to males and has no effect on female fruit flies.

Amino Acid Series.—The diethyl esters in both amino acid series and the dichloro derivative in the methionine series are relatively less toxic to both sexes since about 45% of the males and about 87% of the female fruit flies survived.

The 0.01% aqueous solutions of all the compounds are more toxic to the male flies with the exception of the isopropyl ester (ethylamine series), ethoxychloro ester (methionine series), and dichloro derivative (proline series) which apparently are not toxic.

Received April 28, 1961, from the College of Pharmacy, University of Illinois, Chicago.
Acccepted for publication August 21, 1961.
This paper is abstracted in part from a dissertation submitted to the Graduate College at the Chicago Professional Colleges of the University of Illinois by Florence C. Klee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmaceutical chemistry.
Presented to the Scientific section A.PH.A., Chicago meeting, April 1961.
The authors wish to thank Dr. Katsuyuki Yokoyama for his suggestions concerning the culturing and handling of large numbers of fruit flies. The authors also wish to thank Dr. Halina Presley for Drosophila cultures consisting of two stocks, wild or red eye, and white eye.
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TABLE I.—PER CENT EXPIRED OF FRUIT FLIES IN 24 HOURS

No.	Compound[a]	Concentration, % w/v: 0.1 Total	0.1 Male	0.1 Female	0.01 Total	0.01 Male	0.01 Female	0.001 Total	0.001 Male	0.001 Female
1	$X(OC_2H_5)_2$	100	100	100	15	28	2	7	14	0
2	$X(OCH_2CH_2CH_3)_2$	93	96	90	9	12	6	0	0	0
3	$X(OCH(CH_3)_2)_2$	100	100	100	2	4	0	0	0	0
4	$X(OCH_2CH_2CH_2CH_3)_2$	96	100	92	19	32	6	6	8	4
5	$X(OCH(CH_3)CH_2CH_3)_2$	100	100	100	47	88	6	4	4	4
6	$X(OC(CH_3)_3)_2$	83	100	66	37	54	20	18	26	10
7	$X(OCH_2CH(CH_3)_2)_2$	63	100	26	25	46	4	7	10	4
8	$X(OC_6H_5)_2$	18	32	4	17	30	4	11	20	2
9	$X(OC_2H_5)Cl$	100	100	100	8	14	2	1	2	0
10	XCl_2	75	90	60	5	10	0	0	0	0
11	$Y(OC_2H_5)_2$	34	58	10	9	10	8	3	4	2
12	$Y(OC_2H_5)Cl$	100	100	100	0	0	0	2	0	4
13	YCl_2	26	46	6	19	32	6	11	18	4
14	$Z(OC_2H_5)_2$	42	62	22	7	12	2	1	2	0
15	$Z(OC_2H_5)Cl$	100	100	100	14	26	2	0	0	0
16	ZCl_2	94	96	92	0	0	0	1	2	0
17	$(C_2H_5O)_2P(S)Cl$[b]	100	100	100	5	10	0	0	0	0

[a] X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N$-P(S). For control purposes, distilled water was used instead of an aqueous suspension or solution of a compound under test; it was observed that both sexes survived after 24 hours. [b] Reference standard.

Both sexes are unaffected by 0.001% aqueous solutions of the compounds with the exception of five derivatives in the ethylamine series (phenyl, ethyl, normal, tertiary, and isobutyl esters) and the dichloro derivative in the methionine series which are somewhat toxic to the male flies.

DISCUSSION

The biological responses of the fruit flies indicate to the authors that these compounds are acetylcholinesterase inhibitors.

Concentration-Toxicity Relationship

Varying the Concentration of Compounds in the Ethylamine Series, $C_2H_5N(H)-P(S)(X)_2$.[1]—The greatest decrease in toxicity after one tenfold dilution of the 0.1% w/v aqueous suspension or solution is observed with the two and three carbon chain esters and no change in toxicity is observed with the phenyl ester (Table I). The ethoxychloro, ethyl, and normal propyl esters are 87% as effective, whereas the isopropyl ester is no longer toxic to fruit flies. Following a second tenfold dilution, only the diethyl and phenyl esters are somewhat toxic (about 17%) to males.

The four carbon straight-chain ester (normal butyl) and the dihalo derivative are about 74 and 9% as effective after each tenfold dilution.

Only a 46% decrease in toxicity is observed upon one tenfold dilution of the 0.1% suspensions of the four carbon branched-chain esters. After the second tenfold dilution, the decrease in toxicity is 43% for the secondary butyl ester, and about 18% for the tertiary and isobutyl esters. The most toxic compound in 0.01% solution is the *sec*-butyl ester, and the *tert*-butyl ester is the most lethal in 0.001% solution.

Varying the Concentration of Compounds in the DL-Methionine Series, DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)(X)_2$.[2]—The ethoxychloro derivative is not toxic to flies after one tenfold dilution of the 0.1% suspension.

The dihalo derivative exhibits a 7% decrease in toxicity for each tenfold dilution of the 0.1% suspension and is toxic to males in 0.01% and 0.001% solutions.

The diethoxy ester, which is slightly toxic to both sexes as a 0.01% solution, decreases in toxicity 25 and 6% per tenfold dilution of the 0.1% suspension.

Varying the Concentration of Compounds in the L-Proline Series L-$CH_2CH_2CH_2CHN(CO_2C_2H_5)-P(S)(X)_2$.[2]—The order of decreasing toxicity upon one tenfold dilution is the dichloro derivative (94%), followed by the ethoxychloro (86%), and diethoxy (35%) esters. The latter two esters are toxic to males as 0.01% solutions, the ethoxychloro ester being twice as potent as the diethoxy ester.

These compounds are not toxic when a second tenfold dilution is made.

Structure-Toxicity Relationship

Varying X[1] Groups: Ethylamine Series $C_2H_5N(H)-P(S)(X)_2$.—Observations of the results obtained with the 0.1% suspensions or solutions indicate that with the exception of the isobutyl and concentration %, w/v phenyl esters, all of the esters are more toxic than the dihalo derivative (Table II).

The compounds display a greater range of toxicity when tested as 0.01% solutions. Branching of the four carbon atom chain enhances toxicity to fruit flies since the four carbon branched-chain esters are more toxic than the corresponding straight-chain ester.

[1] $(X)_2$ = $(O\text{-Alkyl})_2$, $(O\text{-}C_6H_5)_2$, $(C_2H_5O)Cl$, or $(Cl)_2$.

[2] $(X)_2$ = $(OC_2H_5)_2$, $(OC_2H_5)Cl$, or $(Cl)_2$.

TABLE II.—TOTAL PER CENT EXPIRED OF FRUIT FLIES IN 24 HOURS,[a] VARYING THE GROUPS[b] ATTACHED TO THE AMINE[c]-P(S) NUCLEUS

Concentration %, w/v					
0.1		0.01		0.001	
Compound[d]	Total	Compound[d]	Total	Compound[d]	Total
$X(OC_2H_5)_2$	100	$X(OCH(CH_3)CH_2CH_3)_2$	47	$X(OC(CH_3)_3)_2$	18
$X(OCH(CH_3)_2)_2$	100	$X(OC(CH_3)_3)_2$	37	$X(OC_6H_5)_2$	11
$X(OCH(CH_3)CH_2CH_3)_2$	100	$X(OCH_2CH(CH_3)_2)_2$	25	$X(OC_2H_5)_2$	7
$X(OC_2H_5)Cl$	100	$X(OCH_2CH_2CH_2CH_3)_2$	19	$X(OCH_2CH(CH_3)_2)_2$	7
$X(OCH_2CH_2CH_2CH_3)_2$	96	$X(OC_6H_5)_2$	17	$X(OCH_2CH_2CH_2CH_3)_2$	6
$X(OCH_2CH_2CH_3)_2$	93	$X(OC_2H_5)_2$	15	$X(OCH(CH_3)CH_2CH_3)_2$	4
$X(OC(CH_3)_3)_2$	83	$X(OCH_2CH_2CH_3)_2$	9	$X(OC_2H_5)Cl$	1
XCl_2	75	$X(OC_2H_5)Cl$	8	XCl_2	0
$X(OCH_2CH(CH_3)_2)_2$	63	XCl_2	5	$X(OCH_2CH_2CH_3)_2$	0
$X(OC_6H_5)_2$	18	$X(OCH(CH_3)_2)_2$	2	$X(OCH(CH_3)_2)_2$	0
$Y(OC_2H_5)Cl$	100	YCl_2	19	YCl_2	11
$Y(OC_2H_5)_2$	34	$Y(OC_2H_5)_2$	9	$Y(OC_2H_5)_2$	3
YCl_2	26	$Y(OC_2H_5)Cl$	0	$Y(OC_2H_5)Cl$	2
$Z(OC_2H_5)Cl$	100	$Z(OC_2H_5)Cl$	14	$Z(OC_2H_5)_2$	1
ZCl_2	94	$Z(OC_2H_5)_2$	7	ZCl_2	1
$Z(OC_2H_5)_2$	42	ZCl_2	0	$Z(OC_2H_5)Cl$	0
$(C_2H_5O)_2P(S)Cl$[e]	100	$(C_2H_5O)_2P(S)Cl$[e]	5	$(C_2H_5O)_2P(S)Cl$[e]	0

[a] The compounds at each dilution are arranged according to decreasing toxicity. [b] (O-Alkyl)$_2$, (O-C_6H_5)$_2$, (OC_2H_5)Cl, or (Cl)$_2$. [c] Ethylamine, DL-methionine, or L-proline ethyl esters. [d] X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N$-P(S). [e] Reference standard.

Apparently there is no differentiation as far as toxicity to male flies is concerned when ethoxy or phenoxy groups are substituted for the normal



butoxy groups attached to the C_2H_5N— nucleus (Tables I and II). These esters are toxic to male fruit flies to the extent of 30%.

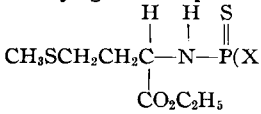
The normal propyl ester is somewhat toxic (12%) to male flies. Branching of this chain, unlike the butyl series, causes a decrease in toxicity and the resulting isopropyl ester is not toxic to flies.

The ethoxychloro and dichloro derivatives are as toxic to male flies as the normal propyl ester.

The tertiary butyl ester, representing maximum branching of a four carbon chain is the most toxic compound to both sexes in 0.001% solution, and the secondary butyl, halo, and three carbon chain derivatives are the least toxic since the latter have no effect on flies at this concentration.

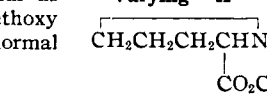
The normal and isobutyl esters are about one-third as toxic and the phenyl and ethyl esters are about two-thirds as toxic to males as the tertiary butyl ester.

Varying X[2] Groups: DL-Methionine Series, DL-

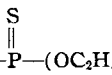


—The results obtained with the 0.1% suspensions of the compounds indicate that when C_2H_5O, Cl groups are attached to this nucleus, maximum toxicity to both sexes results since there are no survivors (Tables I and II). The replacement of the halogen atom by an ethoxy group causes a 90% loss in toxicity to the females and a 42% decrease in toxicity to males. Similarly, when two chlorine atoms are attached to this nucleus the compound is not toxic to females and there is a 54% decrease in toxicity to males. The dichloro derivative is toxic to males to the extent of 18% even after two tenfold dilutions.

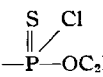
Varying X[2] Groups: L-Proline Series, L-



—In contrast to what is observed in the methionine series, equally potent derivatives are obtained when C_2H_5O, Cl groups or two Cl atoms are attached to this nucleus. There are practically no survivors of either sex. Two ethoxy groups on this nucleus produces a decrease in the toxicity to the males and females of about 40 and 80%, respectively. The toxicity of the dichloro derivative is lost upon further dilution. The diethoxy and ethoxychloro derivatives are no longer toxic to females upon one tenfold dilution, but weakly toxic to males.

Varying the Amine:[3]  **Nucleus.**—When the amine attached to this nucleus is ethylamine, an ester is obtained which is quite toxic to both male and female flies when tested in a 0.1% solution and somewhat toxic to males after two tenfold dilutions (Table III). Substituting methionine or proline for ethylamine results in approximately 40% loss in toxicity to males and about 84% loss in toxicity to females. The 0.01% solutions of the amino acid esters are somewhat toxic to male fruit flies but have no effect on flies after a tenfold dilution.

Apparently, substituting methionine for proline makes very little difference in the toxicity of the ester obtained, but substituting ethylamine for either amino acid enhances its toxicity.

Varying the Amine:[3]  **Nucleus.**—All of the amines produce esters which are equally potent in 0.1% suspensions or solutions and nontoxic in 0.001% solutions.

[3] Ethylamine or the DL-methionine and L-proline ethyl esters.

TABLE III.—PER CENT EXPIRED OF FRUIT FLIES IN 24 HOURS VARYING THE AMINE[a] ATTACHED TO THE —P(S)(X)$_2$[b] NUCLEUS

Amine[a]	Concentration, % w/v: 0.1 Total %	0.1 Male %	0.1 Female %	0.01 Total %	0.01 Male %	0.01 Female %	0.001 Total %	0.001 Male %	0.001 Female %
				P(S)(OC$_2$H$_5$)$_2$ Nucleus					
Ethylamine	100	100	100	15	28	2	7	14	0
Methionine	34	58	10	9	10	8	3	4	2
Proline	42	62	22	7	12	2	1	2	0
				P(S)(OC$_2$H$_5$)Cl Nucleus					
Ethylamine	100	100	100	8	14	2	1	2	0
Methionine	100	100	100	0	0	0	2	0	4
Proline	100	100	100	14	26	2	0	0	0
				P(S)Cl$_2$ Nucleus					
Ethylamine	75	90	60	5	10	0	0	0	0
Methionine	26	46	6	19	32	6	11	18	4
Proline	94	96	92	0	0	0	1	2	0

[a] Ethylamine or the DL-methionine and L-proline ethyl esters. [b] (X)$_2$ = (OC$_2$H$_5$)$_2$, (OC$_2$H$_5$)Cl, or (Cl)$_2$.

A difference in toxicity can be noted only with the 0.01% solutions where L-proline produces a derivative more toxic than that of ethylamine or DL-methionine. The methionine derivative is not toxic at this concentration.

Varying the Amine:[3] —P(S)(Cl)$_2$ **Nucleus.**—The order of decreasing toxicity in 0.1% suspensions or solutions is proline, followed by ethylamine, and then methionine. When proline is attached to this nucleus, a derivative results which is toxic to both sexes. Replacement of proline by ethylamine produces a derivative which retains the same toxicity to males but is about 30% less toxic to females. Methionine attached to this nucleus produces a derivative which is not toxic to females and, compared to the proline derivative, is 50% less toxic to males.

The order of decreasing toxicity for the 0.01% solutions is reversed, that is, methionine, followed by ethylamine, and then proline, because ethylamine is only weakly toxic to males and proline is no longer toxic to flies at this concentration.

It is interesting to observe that the methionine derivative in 0.001% solution retains its toxicity to males to the extent of 18%.

SUMMARY

Biological responses of the fruit flies indicate that the thiophosphoric acid derivatives are anticholinesterases.

Results on mature fruit flies show that neither sex survives in 0.1% w/v suspensions of most of the derivatives. The 0.01% solutions are more toxic to the male flies than to the females. Both sexes are unaffected by 0.001% solutions, with the exception of five esters of the ethylamine series (*n*-propyl, *n*-, *tert*-, isobutyl, and phenyl esters), and one methionine derivative (dichloro), which were somewhat toxic to the male flies.

Concentration-toxicity and structure-toxicity relationships are discussed.

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(1) Klee, F. C., and Kirch, E. R., THIS JOURNAL, **51**, 423(1962).
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Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters IV

Biological Activities in Seeds of *Hyoscyamus niger*

By FLORENCE C. KLEE† and ERNST R. KIRCH

The effect of certain thiophosphoric acid derivatives on germination and seedling growth of hyoscyamus seeds was observed. All of the compounds as 0.1% w/v suspensions or solutions inhibited the growth of the seedlings. Four ethylamine and two DL-methionine derivatives inhibited germination, whereas two esters stimulated germination. Inhibition and stimulation of germination and growth were observed with some of the 0.01% solutions. The 0.001% solutions had no effect on germination, but seven promoted the growth of the seedling. Structure-toxicity relationship is discussed.

THE SYNTHESIS of thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters (1) and their biological activities in guppies (2) and fruit flies (3) have been reported recently.

These thiophosphoric acid derivatives have structures similar to known anticholinesterases.

$$\begin{array}{c} \quad\quad S \\ | \quad \| \\ -N-P(X)_2 \end{array}$$

Their general formula is —N—$P(X)_2$, where N represents the amine or amino acid ester moiety and $(X)_2$ = $(O\text{-alkyl})_2$, $(O\text{-}C_6H_5)_2$, $(C_2H_5O)Cl$, or $(Cl)_2$. The observed biological responses of guppies and fruit flies which have been in contact with aqueous solutions of these compounds seem to indicate that these compounds are indeed acetylcholinesterase inhibitors.

There exists the possibility of these thiophosphoric acid derivatives inhibiting other enzymes since Aldridge has shown that organophosphorus compounds are not specific inhibitors for the cholinesterases but rather inhibitors for enzymes possessing carboxylic esterase activity (4). Seeds contain many enzymes playing a role in the synthesis and hydrolysis of carbohydrates, proteins, and lipids (5). The purpose of this investigation is to observe the effect of 0.1, 0.01, and 0.001% w/v aqueous suspensions or solutions of certain thiophosphoric acid derivatives on the germination and seedling growth of *Hyoscyamus niger* L. seeds.

Received April 28, 1961, from the College of Pharmacy, University of Illinois, Chicago.

Accepted for publication August 21, 1961.

This paper is abstracted in part from a dissertation submitted to the Graduate College at the Chicago Professional Colleges of the University of Illinois by Florence C. Klee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmaceutical chemistry.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

The authors wish to thank Dr. Katsuyuki Yokoyama for the supply of *Hyoscyamus niger* seeds for this investigation. The authors are grateful to Dr. Allan M. Burkman for his helpful suggestions concerning statistical calculations.

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EXPERIMENTAL

Two milliliters of an aqueous suspension or solution (0.1, 0.01, and 0.001% w/v) of each compound in Table I was slowly introduced with a syringe on a filter paper containing 80 seeds (*Hyoscyamus niger*) in a 9-cm. Petri dish. For a control, two ml. of distilled water was used instead of the aqueous solution of the compound to be tested.

The Petri plates were covered and placed in the dark for 2 days. On the second day, two ml. of distilled water was introduced into each dish. After 4 days, the number of seedlings which had germinated was recorded and the length of each seedling was measured.

Table I shows the per cent germination, the average height of the seedling observed for each compound at three concentrations, and the standard deviation. The percentages of inhibition or stimulation of germination and seedling growth as compared to the control are presented in Tables II and III, respectively.

Germination.—Germination of the seeds was inhibited to various extents by 0.1% w/v aqueous suspensions or solutions of six compounds, four of which are in the ethylamine series and two in the DL-methionine series (Table II). The dichloro derivative in the ethylamine series is the strongest inhibitor (86%), whereas the diethyl ester (ethylamine series) and dihalo derivative of the methionine series are only half as effective. The ethoxychloro derivative (ethylamine series) is much less effective (16% inhibition), and the weakest inhibition is observed with the tertiary butyl ester (ethylamine series) and the diethyl ester in the methionine series (about 9% inhibition).

It was interesting to observe that the secondary butyl and phenyl esters in the ethylamine series stimulated germination to the extent of 10 and 5%, respectively.

The 0.01% solution of diethoxyethylamidothiophosphate is 17% inhibitory, whereas the diethyl ester in the methionine series is a weak inhibitor (6% inhibition). The normal butyl ester stimulated germination to the extent of 10%.

The 0.001% solutions of the compounds had no effect on the germination.

Growth.—All of the compounds as 0.1% suspensions or solutions inhibited the growth of the *Hyoscyamus niger* seedlings (Table III). Maximum

TABLE I.—EFFECT OF VARIOUS DERIVATIVES OF THIOPHOSPHORIC ACID ON GERMINATION AND SEEDLING GROWTH OF HYOSCYAMUS SEEDS

	Concentration, % w/v											
	0			0.001			0.01			0.1		
Compound[a]	Germination, %	Median Seedling Height, mm.	±S.D.[b]	Germination, %	Median Seedling Height, mm.	±S.D.[b]	Germination, %	Median Seedling Height, mm.	±S.D.[b]	Germination, %	Median Seedling Height, mm.	±S.D.[b]
$X(OC_2H_5)_2$	90.00	24.8	4.8	93.75	23.4	3.6	75.00	19.2	5.1	50.00	4.5	2.0
$X(OCH_2CH_2CH_3)_2$				87.50	25.8	5.1	93.75	23.3	3.4	87.50	4.9	1.1
$X(OCH(CH_3)_2)_2$				93.75	20.0	3.8	86.25	18.4	3.8	82.50	13.4	3.7
$X(OCH_2CH_2CH_2CH_3)_2$	88.75	15.9	4.0	90.00	19.7	4.1	97.50	15.3	3.3	96.25	7.9	1.9
$X(OCH(CH_3)CH_2CH_3)_2$				83.75	17.9	2.5	92.50	17.6	3.6	97.50	6.8	1.7
$X(OC(CH_3)_3)_2$				95.00	17.5	3.4	88.75	14.3	3.9	86.25	6.2	3.1
	95.00	17.6	4.0									
$X(OCH_2CH(CH_3)_2)_2$				91.25	17.5	4.7	97.50	15.1	2.5	96.25	7.3	2.1
$X(OC_6H_5)_2$	95.00	23.6	6.9	97.50	23.0	5.8	88.75	20.9	7.1	100.00	19.3	6.3
$X(OC_2H_5)Cl$	88.75	14.0	5.0	91.25	13.3	6.0	93.75	13.5	4.0	75.00	0.32	0.2
XCl_2				93.75	25.7	4.6	100.00	19.2	3.7	13.75	1.2	0.3
	97.50	23.1	5.1									
$(C_2H_5O)_2P(S)Cl$[c]				100.00	23.8	5.3	96.25	20.6	5.4	100.00	0.83	0.5
$Y(OC_2H_5)_2$				96.25	20.7	3.5	93.75	16.4	4.1	91.25	14.4	3.6
	100.00	17.3	3.3									
$Z(OC_2H_5)_2$				100.00	19.8	5.2	97.50	18.5	4.3	97.50	11.3	4.0
$Y(OC_2H_5)Cl$				98.75	24.0	4.4	97.50	24.1	4.6	96.25	18.0	2.9
	98.75	24.7	5.7									
YCl_2				98.75	23.7	4.9	98.75	21.7	3.4	58.75	0.73	0.3
$Z(OC_2H_5)Cl$				95.00	23.2	4.4	95.00	20.3	4.3	98.75	13.3	2.9
	95.00	22.3	5.7									
ZCl_2				95.00	24.0	4.2	95.00	19.5	5.3	98.75	3.5	3.1

[a] X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N$-P(S). [b] S.D. = Standard deviation. [c] Reference standard.

TABLE II.—PERCENTAGES OF INHIBITION OR STIMULATION OF GERMINATION OF HYOSCYAMUS SEEDS

Compound[a]	Concentration, % w/v[b] 0.001	0.01	0.1
$X(OC_2H_5)_2$	(+)4.2	(−)16.7[c]	(−)44.4[d]
$X(OCH_2CH_2CH_3)_2$	(−)2.8	(+) 4.2	(−) 2.8
$X(OCH(CH_3)_2)_2$	(+)5.6	(−) 2.8	(−) 7.0
$X(OCH_2CH_2CH_2CH_3)_2$	(+)1.4	(+) 9.9[e]	(+) 8.5
$X(OCH(CH_3)CH_2CH_3)_2$	(−)5.6	(+) 4.2	(+) 9.9[e]
$X(OC(CH_3)_3)_2$	0.0	(−) 6.6	(−) 9.2[e]
$X(OCH_2CH(CH_3)_2)_2$	(−)4.0	(+) 2.6	(+) 1.3
$X(OC_6H_5)_2$	(+)2.6	(−) 6.6	(+) 5.3[e]
$X(OC_2H_5)Cl$	(+)2.8	(+) 5.6	(−)15.5[e]
XCl_2	(−)3.9	(+) 2.6	(−)85.9[d]
$Y(OC_2H_5)_2$	(−)3.8	(−) 6.3[e]	(−) 8.8[c]
$Y(OC_2H_5)Cl$	0.0	(−) 1.3	(−) 2.5
YCl_2	0.0	0.0	(−)40.5[d]
$Z(OC_2H_5)_2$	0.0	(−) 2.5	(−) 2.5
$Z(OC_2H_5)Cl$	0.0	0.0	(+) 4.0
ZCl_2	0.0	0.0	(+) 4.0
$(C_2H_5O)_2P(S)Cl$	(+)2.6	(−) 1.3	(+) 2.6

[a] X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)$. [b] (+) = % germination greater than control (% stimulation); (−) = % germination less than control (% inhibition). [c] Significant at the 1% level. [d] Significant at the 0.1% level. [e] Significant at the 5% level.

TABLE III.—PERCENTAGES OF INHIBITION OR STIMULATION OF GROWTH OF THE *Hyoscyamus niger* SEEDLING

Compound[a]	Concentration, % w/v[b] 0.001	0.01	0.1
$X(OC_2H_5)_2$	(−) 5.6[c]	(−)22.6[d]	(−)81.9[d]
$X(OCH_2CH_2CH_3)_2$	(+) 4.0	(−) 6.0[c]	(−)80.2[d]
$X(OCH(CH_3)_2)_2$	(+)25.8[d]	(+)15.7[d]	(−)15.7[d]
$X(OCH_2CH_2CH_2CH_3)_2$	(+)24.0[d]	(−) 3.8	(−)50.3[d]
$X(OCH(CH_3)CH_2CH_3)_2$	(+)12.6[d]	(+)10.7[c]	(−)57.2[d]
$X(OC(CH_3)_3)_2$	(−) 0.6	(−)18.8[d]	(−)64.8[d]
$X(OCH_2CH(CH_3)_2)_2$	(−) 0.6	(−)14.2[d]	(−)58.5[d]
$X(OC_6H_5)_2$	(−) 2.5	(−)11.4[c]	(−)18.2[d]
$X(OC_2H_5)Cl$	(−) 5.0	(−) 3.6	(−)97.9[d]
XCl_2	(+)11.3[d]	(−)16.9[d]	(−)94.8[d]
$Y(OC_2H_5)_2$	(+)19.7[d]	(−) 5.2	(−)16.8[d]
$Y(OC_2H_5)Cl$	(−) 2.8	(−) 2.4	(−)27.1[d]
YCl_2	(−) 4.0	(−)12.2[d]	(−)97.0[d]
$Z(OC_2H_5)_2$	(+)14.4[d]	(+) 6.9[c]	(−)34.7[d]
$Z(OC_2H_5)Cl$	(+) 4.0	(−) 9.0[c]	(−)40.4[d]
ZCl_2	(+) 7.6[c]	(−)12.6[d]	(−)84.3[d]
$(C_2H_5O)_2P(S)Cl$	(+) 3.0	(−)10.8[d]	(−)96.5[d]

[a] X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)$. [b] (+) = % growth greater than control (% stimulation); (−) = % growth less than control (% inhibition). [c] Significant at the 5% level. [d] Significant at the 0.1% level. [e] Significant at the 1% level.

growth inhibition (average of 94%) was caused by the ethoxychloro ester in the ethylamine series and all of the dihalo derivatives.

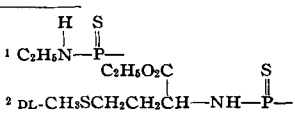
Almost all of the 0.01% solutions of compounds in the three series had an effect on seedling growth. Maximum inhibition (about 20%) was observed with derivatives in the ethylamine series, namely, the diethyl, tertiary butyl, and dichloro derivatives. Stimulation of seedling growth was caused by two esters in the ethylamine series, isopropyl (16%), secondary butyl (11%), and the diethyl ester (7%) in the proline series.

Seven of the 0.001% solutions promoted the growth of the seedling. In the ethylamine series, the isopropyl and normal butyl esters stimulated the seedling growth to the extent of 25%, and the secondary butyl and dichloro derivatives were 12% stimulatory. The diethyl esters in both amino acid series promoted growth to about 17%, whereas the dichloro derivative in the proline series was about 9% less stimulatory. Only one ester, diethoxyethylamidothiophosphate, inhibited seedling growth to the extent of 6%.

DISCUSSION

The effect of various derivatives of thiophosphoric acid on the germination and seedling growth of seeds of *Hyoscyamus niger* was primarily inhibition, however, some stimulation was also observed (Tables II and III).

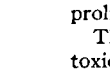
Structure-Toxicity Relationship.—The effects of the 0.1% suspensions or solutions on the germination of hyoscyamus seeds indicate that derivatives which have two chlorine atoms or two ethoxy groups attached to the ethylamine[1] or methionine[2] nucleus



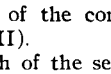
cause inhibition of germination. The ethylamine derivatives are more potent than the corresponding methionine derivatives. The ethoxychloro and tertiary butyl esters in the ethylamine series are weak inhibitors. It is interesting to note that the proline derivatives have no effect on germination.

The following relationships between structure and toxicity are based on the results obtained with the 0.1% suspensions or solutions of the compounds on the seedling growth (Table III).

Varying the Amine.[3]—Growth of the seedling is inhibited to the extent of about 92% irrespective of the particular amine that is attached to the

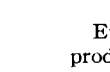


—$P(Cl)_2$ nucleus.



Ethylamine attached to the —$P(=S)(Cl)OC_2H_5$ nucleus produces a derivative which is 98% inhibitory to growth. When ethylamine is replaced by methionine or proline, much of the inhibitory effect of the derivative is lost since the two amino acid derivatives inhibit growth only to the extent of about 34%.

The amine imparting the most toxicity to the



—$P(OC_2H_5)_2$ nucleus is ethylamine (82% inhibition). A 47% decrease in inhibition of growth is observed when proline is substituted for ethylamine. The methionine derivative is 65% less inhibitory than the ethylamine derivative.

Varying the $(X)_2$ Groups[4]



C_2H_5N—P— **Nucleus.**—The order of decrease in the inhibition of growth when the X groups of this nucleus are varied is mono and dichloro (96%), ethyl, *n*-propyl (81%), tertiary (65%), secondary, isobutyl (58%), *n*-butyl (50%), phenyl, and isopropyl (17%).

The halogen derivatives are the most potent growth inhibitors. Two and three carbon straight-chain esters also are very effective; however, the three carbon branched-chain ester has much less inhibitory effect on the growth of the seedling.

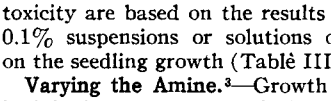
Unlike the three carbon esters, branching of the four carbon chain esters enhances the inhibitory effect since the branched-chain esters are more inhibitory to growth than the corresponding straight-chain ester.

Replacement of the two and three carbon straight-chains by a four carbon straight-chain results in an ester which is 31% less inhibitory.

A phenyl ring in place of a halogen or an alkyl group (with the exception of a three carbon branched-chain) greatly decreases the inhibitory action of the compound.



DL-$CH_3SCH_2CH_2C$—N—P— **Nucleus.**—In the methionine series, two chlorine atoms attached to this nucleus produces a derivative which inhibits seedling growth to the extent of 97%. The replacement of one chlorine atom with an ethoxy group results in a 70% loss in the inhibitory action, and when both chlorine atoms are replaced, 80% of this effect is lost.



L-$CH_2CH_2CH_2CHN$—P— **Nucleus.**—As was observed in the methionine series, two chlorine atoms on this nucleus result in a potent growth-inhibitory substance. The inhibitory effect is greatly diminished (by more than 40%) upon substitution of the halogen atoms with one or two ethoxy groups.

[3] Ethylamine or the DL-methionine and L-proline ethyl esters.

[4] $(X)_2$ = $(O\text{-Alkyl})_2$, $(O\text{-}C_6H_5)_2$, $(C_2H_5O)Cl$, or $(Cl)_2$.

SUMMARY

Primarily, inhibition and, to some extent, stimulation were the effects of certain derivatives of thiophosphoric acid on the germination and seedling growth of the seeds of *Hyoscyamus niger*.

Seed germination was inhibited to various extents by the 0.1% suspensions of four ethylamine (dichloro, diethoxy, ethoxychloro, and tertiary butyl) and two methionine (dichloro and diethoxy) derivatives. In contrast, germination was stimulated by three esters, the 0.1% suspensions of the secondary butyl and phenyl esters, and the 0.01% solution of the normal butyl ester. The 0.01% solutions of the diethyl esters in the ethylamine and methionine series had an inhibitory effect. The 0.001% solutions of all the compounds had no effect on germination. Germination was unaffected by any of the solutions of the proline derivatives.

All of the compounds as 0.1% suspensions or solutions inhibited, to various extents, the growth of the seedling. Maximum growth inhibition was caused by the ethoxychloro ester in the ethylamine series and all of the dihalo derivatives. Ten of the 0.01% solutions had an inhibitory effect and three of the solutions had a stimulatory effect on seedling growth. Growth was promoted by seven of the 0.001% solutions.

The relationship between structure and toxicity is discussed.

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Comparison of Anticonvulsant and Psychopharmacologic Drugs

By GREGORY B. FINK† and EWART A. SWINYARD

Four anticonvulsant drugs were compared with seven psychopharmacologic agents by a battery of tests designed to detect possible tranquilizing activity. The drugs tested were found to have only one property in common, the ability to prolong hexobarbital sleep time. Five psychopharmacologic agents, chlorpromazine, promazine, trifluprom azine, reserpine, and hydroxyzine, blocked conditioned avoidance responses and reduced amphetamine toxicity in aggregated mice, whereas the two sedative anticonvulsants, phenobarbital and trimethadione, and two psychopharmacologic agents, meprobamate and phenaglycodol, were inactive by these tests. The two nonsedative anticonvulsants, diphenylhydantoin and phenacemide, markedly reduced amphetamine toxicity in aggregated mice, which suggests that these two anticonvulsant drugs may have mild tranquilizing properties.

IN PREVIOUS reports from our laboratories, the anticonvulsant activity of several psychopharmacologic drugs with tranquilizing properties were compared with those of conventional antiepileptic agents (1, 2). In the present report, the tranquilizing activities of four antiepileptic drugs are compared with those of some clinically useful psychopharmacologic agents. This study was prompted by the fact that some conventional anticonvulsant drugs not only control epileptic seizures, but also may exert a salutary effect on the behavioral disturbances which often accompany convulsant disorders (3). Furthermore, anticonvulsant drugs have been used successfully in the treatment of nonepileptic, mentally ill patients (3–7). These observations suggest that anticonvulsant drugs may possess some central properties which are common to those of the psychopharmacologic agents. The experiments described in this communication were designed to test this hypothesis.

METHODS

Adult male albino mice (CF No. 1 strain) obtained from the Carworth Farms were used as experimental animals. They were allowed free access to food and water except during the experimental test period. All drugs were given orally in aqueous solution, except that phenacemide, trimethadione, meprobamate, and phenaglycodol were given as suspensions in 6% acacia solution. The mean neurotoxic dose (TD_{50}) and the time of peak activity were determined for each drug as described elsewhere (2). Equitoxic doses (multiples of the TD_{50}) were given to groups of animals as described below, and the animals subjected to the particular test at the time of peak drug activity. The various drugs examined, their times of peak activity, and their TD_{50}s are listed in Table I.

TABLE I.—TIME OF PEAK EFFECT AND NEUROTOXIC DOSES (TD_{50}) OF SOME ANTICONVULSANT AND PSYCHOPHARMACOLOGIC DRUGS IN MICE

Drug	Time of Peak Drug Effect, min.	Neurotoxic Dose[a] (TD_{50})
Diphenylhydantoin sodium (Dilantin sodium)	180	84 (74–95)
Phenacemide (Phenurone)	45	660 (545–799)
Phenobarbital sodium	180	70 (58–84)
Trimethadione (Tridione)	120	1150 (1055–1253)
Meprobamate (Equanil; Miltown)	30	228 (196–262)
Phenaglycodol (Ultran)	60	170 (119–241)
Hydroxyzine hydrochloride (Atarax hydrochloride)	30	490 (434–553)
Reserpine	240	16.5 (11.6–23.4)
Chlorpromazine hydrochloride (Thorazine hydrochloride)	90	15.7 (10.6–23.2)
Promazine hydrochloride (Sparine hydrochloride)	60	23.5 (15.0–36.9)
Trifluprom azine hydrochloride (Vesprin)	90	7.8 (5.3–11.5)

[a] Values expressed in mg./Kg. with 95% fiducial limits in parentheses.

Hexobarbital Potentiation.—The drugs were administered to groups of 8 mice each. At the time of peak drug effect, hexobarbital sodium (100 mg./Kg.) was injected intravenously. The sleeping time, in minutes, was measured from the end of the injection until the animal regained its righting reflex. The per cent increase in the sleep time of the drug-treated group over that of the corresponding control group run concurrently was calculated and the results were analyzed by the *t* test.

Received September 8, 1961, from the Departments of Pharmacology, University of Utah, College of Pharmacy and College of Medicine, Salt Lake City.

Accepted for publication September 28, 1961.

This investigation was supported by a research grant from the National Institute of Neurological Diseases and Blindness (B-381), National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

This paper is a portion of a thesis submitted by Gregory B. Fink in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Utah.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

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Conditioned Response Test.—A shuttle box (20 in. by 4 in. by 11 in.) was divided into two compartments (one black and one white) of equal length and width, by a barrier formed by elevating the smooth white floor of the white compartment 2 in. The black compartment had walls 9 in. high and a grid floor composed of stainless steel rods (1/8 in. diameter, placed 1/4 in. apart) through which an electric shock (60-cycle a.c.; 50 v., delivered through a grid scrambler) could be applied to the feet of the mouse. An electric buzzer was placed beneath the grid floor of the black compartment and a 25-w. light bulb with a reflector was placed above it. Mice were placed individually in the black compartment. After 15-seconds exposure to the environment, the buzzer and light were activated for 10 seconds. An animal could avoid the electric shock that would follow by escaping to the white compartment (conditioned response, CR). Each mouse was further conditioned until it would escape to the white compartment when placed in the box (secondary conditioned response, SCR). Three end points were used to determine drug activity: abolition of (*a*) the SCR (exposure to 15 seconds of the environment), (*b*) the CR (exposure to 15 seconds of environment alone, and 10 seconds of buzzer and light), and (*c*) the unconditioned response (UR, exposure to 15 seconds of environment alone, 10 seconds of buzzer and light, and, finally, 5 seconds of shock associated with the buzzer and light). The response of each mouse was graded according to an arbitrary point system: a mouse exhibiting a SCR was awarded 3 points; a CR, 2 points; a UR, 1 point; nonresponders to 5 seconds of shock were assigned 0 points. Drug activity was determined in groups of 8 mice. Each mouse was given a block of 4 consecutive trials at 1-minute intervals (a fully conditioned mouse would score 12 points in this control response) and then administered the drug to be tested. At selected times after drug administration, the mice were given another block of trials and the responses recorded. A control group was always tested concurrently with the drug-treated groups to insure that the SCR and CR were not extinguished spontaneously, despite the fact that preliminary experiments indicated that there were no significant differences in the total scores of a group of 8 control mice given successive blocks of trials over a 4-hour period. The data were statistically analyzed by the Walsh test (8).

Amphetamine Toxicity Tests.—The drugs were initially tested by the 3-mice-per-cage test described by Lasagna and McCann (9), except that an intraperitoneal dose of 97.5 mg./Kg. of amphetamine, equivalent to 3 times the LD_{50} determined in aggregated mice, was employed as previously reported (10). This test was so severe that even reserpine, a drug known to have tranquilizing properties, was ineffective in nontoxic doses. Therefore, amphetamine toxicity was also studied in aggregated mice by a modification of the 10-mice-per-cage test described by Burn and Hobbs (11). Fifty mice were randomly divided into 5 groups of 10 mice each. A different drug at a selected dose level was administered to each of four groups, and the requisite volume of vehicle was given to the fifth group, which served as a control. Thirty minutes before the time of peak effect for each drug (15 minutes for drugs with a time of peak effect of 30 minutes), each animal was given 25 mg./Kg. of amphetamine sulfate intraperitoneally. Each group of mice was then placed in a clear plastic cage (27 cm. by 17 cm. by 12 cm.) with a screen bottom. Twenty-four hours later the number of dead mice in each box was recorded. The above procedure was repeated until 20 mice (2 groups of 10 mice each) had received the selected dose of each drug. The data were statistically analyzed by the Chi-square method, and drug activity was expressed as a ratio (% of deaths in control group/% of deaths in drug-treated group).

RESULTS

The effects of some anticonvulsant and psychopharmacologic drugs on hexobarbital sleep time in mice are summarized in Table II. As indicated in the table, all of the agents significantly increased hexobarbital sleep time. The increase in sleep time ranged from 40% for meprobamate to 240% for diphenylhydantoin.

TABLE II.—EFFECTS OF SOME ANTICONVULSANT AND PSYCHOPHARMACOLOGIC DRUGS ON HEXOBARBITAL SLEEP TIME IN MICE

Drug	Dose mg./Kg.	Dose Multiples TD_{50}	Increase in Sleep Time,[a] %
Diphenylhydantoin	42	1/2	240
Phenacemide	330	1/2	177
Phenobarbital	35	1/2	53
Trimethadione	575	1/2	81
Meprobamate	114	1/2	40
Phenaglycodol	85	1/2	207
Hydroxyzine	122	1/4	117
Reserpine	8.2	1/2	55
Chlorpromazine	7.8	1/2	184
Promazine	11.7	1/2	71
Trifluprom azine	3.9	1/2	168

[a] All values in this column are significantly different from controls ($P < 0.05$).

The effects of these same drugs on conditioned responses in mice are shown in Table III; these effects are expressed as activity ratios (total points in predrug blocks of trials/total points in postdrug blocks of trials). Ideally, a drug that blocks only the secondary conditioned response (SCR) in all animals has an activity ratio of 1.5 (12/8), whereas a drug that blocks the conditioned response (CR) in all animals has a ratio of 3.0 (12/4). Ratios above 3.0 indicate that the unconditioned response (UR) is also blocked in some animals. The four anticonvulsant drugs (diphenylhydantoin, phenacemide, phenobarbital, and trimethadione) did not significantly suppress either the SCR or the CR even after doses up to the TD_{50}. Meprobamate and phenaglycodol, in doses equivalent to the TD_{50}, partially blocked the SCR, although only the activity ratio of meprobamate, 1.28, was significant. Hydroxyzine at 1/2 the TD_{50} blocked the SCR in all animals, whereas 8.2 mg./Kg. of reserpine (1/2 TD_{50}) abolished the CR in all animals and blocked the UR in some animals, as indicated by an activity ratio of 3.39. Chlorpromazine exhibited greater activity as the dose level was increased. At dose levels of 3.9 and 7.8 mg./Kg., the SCR and the CR were successively

TABLE III.—EFFECTS OF SOME ANTICONVULSANT AND PSYCHOPHARMACOLOGIC DRUGS ON CONDITIONED RESPONSES IN MICE

Drug	Dose mg./Kg.	Dose Multiples TD_{50}	Activity Ratio[a]
Diphenylhydantoin	42	1/2	1.18
	84	1	1.21
Phenacemide	660	1	1.02
	1,320	2	1.02
Phenobarbital	70	1	1.08
	140	2	1.15
Trimethadione	575	1/2	0.96
	1,150	1	1.03
Meprobamate	114	1/2	0.99
	228	1	1.28[b]
Phenaglycodol	170	1	1.22
Hydroxyzine	122	1/4	1.32[b]
	245	1/2	1.64[b]
Reserpine	8.2	1/2	3.39[b]
Chlorpromazine	3.9	1/4	1.35[b]
	7.8	1/2	1.96[b]
	15.7	1	3.10[b]
Promazine	11.7	1/2	1.26[b]
Triflupromazine	3.9	1/2	2.58[b]

[a] Total points in predrug block of trials/total points in postdrug block of trials. [b] Significantly different from controls ($P < 0.05$).

TABLE IV.—EFFECTS OF SOME ANTICONVULSANT AND PSYCHOPHARMACOLOGIC DRUGS ON AMPHETAMINE TOXICITY IN AGGREGATED MICE

Drug	Aggregation Toxicity Test: 3 per Cage (ED_{50})[a]	10 per Cage: Dose mg./Kg.	Multiples (TD_{50})	Ratio[b]
Diphenylhydantoin	>200	42	1/2	7.0[c]
		84	1	4.7[c]
Phenacemide	>1,000	330	1/2	7.0[c]
		660	1	7.0[c]
Phenobarbital	>75	70	1	1.2
Trimethadione	>1,000	575	1/2	0.8
		1,150	1	1.2
Meprobamate	>1,000	228	1	0.8
Phenaglycodol	>1,000	170	1	0.9
Hydroxyzine	>400	175	1/3	2.8[c]
Reserpine	>80	2.0	1/8	4.7[c]
Chlorpromazine	2.0 (1.3–3.2)	2.0	1/8	2.3[c]
Promazine	8.0 (5.0–12.8)	8.0	1/3	2.8[c]
Triflupromazine	3.8 (2.4–6.1)	3.8	1/2	3.5[c]

[a] Values expressed in mg./Kg. with 95% fiducial limits in parentheses. [b] % of deaths in control group/% of deaths in drug-treated group. [c] Significantly different from controls ($P < 0.05$).

suppressed (activity ratios, 1.35 and 1.96, respectively). At the TD_{50} (15.7 mg./Kg.), the drug absolished the CR and occasionally blocked the UR, as indicated by a ratio of 3.10. Trifluprom azine was more active and promazine was less active than chlorpromazine at dose levels equivalent to 1/2 the TD_{50}; the ratios were 2.58, 1.26, and 1.96, respectively.

The effects of some anticonvulsant and psychopharmacologic drugs on amphetamine toxicity in aggregated mice are summarized in Table IV. It is evident that the drugs were less effective against amphetamine toxicity in the 3-mice-per-cage test than in the 10-mice-per-cage test. Except for the three phenothiazine derivatives, all the drugs were ineffective in nontoxic doses by the former test, whereas only phenobarbital, trimethadione, meprobamate, and phenaglycodol were ineffective as measured by the latter test. Thus, chlorpromazine, promazine, and trifluprom azine effectively reduced amphetamine toxicity in both situations, whereas diphenylhydantoin, phenacemide, hydroxyzine, and reserpine effectively reduced the toxicity of this substance only in the less severe 10-mice-per-cage test.

DISCUSSION

Three tests were employed to measure the tranquilizing activity of the drugs selected for study; namely, hexobarbital potentiation, a conditioned avoidance response, and protection against amphetaminetoxicity in aggregated mice. It is well known that many diverse agents, including tranquilizers, sedatives, anesthetics, antihistaminics, and other substances, increase the duration of sleep induced by barbiturates (12). However, this is one of few tests which gives positive results with all drugs claimed to have tranquilizing properties (12). Although conditioned responses are extensively used to evaluate psychotropic substances, the ability to alter conditioned behavior in animals is not limited to tranquilizing agents. For example, morphine, 5-hydroxytryptamine, mescaline, and iproniazid have been shown to block the secondary conditioned response (SCR) and/or conditioned response (CR) (13–15). On the other hand, there have been no reports which indicate that agents other than those with tranquilizing activity can reduce amphetamine toxicity in aggregated mice in nontoxic doses. These facts emphasize that no single method can be relied upon to detect the tranquilizing properties of a drug. The above battery of tests was selected because it appeared broad enough to include all agents with tranquilizing properties and selective enough to delineate drugs with mild tranquilizing activity from those with potent activity.

The data presented indicate that the anticonvulsant and psychopharmacologic drugs studied may be divided into 3 distinct groups: (I) phenobarbital, trimethadione, meprobamate, and phenaglycodol, (II) diphenylhydantoin and phenacemide, and (III) hydroxyzine, reserpine, chlorpromazine, promazine, and trifluprom azine. The drugs in group I are reported to have similar profiles of action (1, 2, 16–18) and all possess sedative-anticonvulsant properties. They are characterized herein by having activity in only one test, hexobarbital potentiation. Thus, this group of drugs probably possesses little tranquilizing activity. The drugs in Group II are nonsedative, anticonvulsant agents and are characterized by ability to potentiate hexobarbital and to reduce amphetamine toxicity in aggregated mice. Thus, the two drugs in this group, diphenylhydantoin and phenacemide, appear to have some properties in common with the drugs in Group III, the tranquilizers, which are characterized by giving positive results in all three tests employed. Of the drugs in Group III, chlorpromazine, promazine, and trifluprom azine appear to be more potent tranquilizers than hydroxyzine and reserpine because they are the only agents

effective by the 3-mice-per-cage amphetamine toxicity test.

The published data on the effects of psychopharmacologic drugs on conditioned avoidance responses in rats are in agreement with those presented herein for mice. In rats, chlorpromazine and reserpine block the SCR and CR (13, 14, 19), whereas hydroxyzine blocks only the SCR (14). Trifluprom azine is more potent (20) and promazine is less potent (14) than chlorpromazine when compared for ability to block the CR in rats. Meprobamate and the barbiturates have little effect on the CR as long as the rats are capable of responding to the UR (electric shock) (13, 16, 19). Because these drugs also have essentially the same profiles of action against conditioned avoidance responses in mice, this species appears to be as useful as rats for evaluating drugs by this technique. In addition, the data reported by Lasagna and McCann (9) and Burn and Hobbs (11) on the effects of psychopharmacologic drugs on amphetamine toxicity in aggregated mice are in agreement with those presented herein. This technique appears to be a useful one for discerning tranquilizing properties in candidate psychotropic substances.

The data indicate that two of the anticonvulsant drugs studied, diphenylhydantoin and phenacemide, may have mild tranquilizing activity. In similarity to the tranquilizers (Group III, above) they lack hypnotic activity and may improve mood and behavior in epileptic patients (3, 21). Clinical reports indicate that diphenylhydantoin also calms disturbed nonepileptic psychotic patients (3–6), conditions in which the phenothiazines are effective (22). Phenacemide may improve behavioral disorders; but, in certain cases, it also causes adverse psychic changes (3, 21, 23). On the basis of these observations, it would appear that diphenylhydantoin and phenacemide do indeed possess some tranquilizing properties and that this property should be investigated more fully in human patients.

SUMMARY

Four anticonvulsant drugs and seven psychopharmacologic agents were tested in mice for ability to increase hexobarbital sleep time, to abolish a conditioned avoidance response (CAR), and to reduce amphetamine toxicity in mice aggregated 3 per cage (more severe test) and 10 per cage (less severe test). All drugs significantly increased hexobarbital sleep time. Chlorpromazine, promazine, trifluprom azine, reserpine, and hydroxyzine blocked the conditioned response and/or secondary conditioned response. Meprobamate, phenaglycodol, and the anticonvulsant drugs (diphenylhydantoin, phenacemide, phenobarbital, and trimethadione) had little effect on CAR in nontoxic doses. Only the phenothiazine derivatives were effective by the 3-per-cage amphetamine toxicity test. The phenothiazines, reserpine, and hydroxyzine were active in the 10-per-cage test; two nonsedative-anticonvulsant drugs, diphenylhydantoin and phenacemide, were also effective by this test, which suggests that they may have some tranquilizing properties. The sedative-anticonvulsant drugs (phenobarbital, trimethadione, meprobamate, and phenaglycodol) were inactive by both amphetamine toxicity tests. The significance of these findings is discussed.

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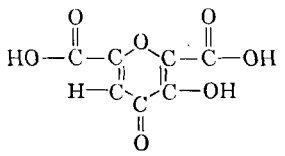
Dissociation Constants of Certain γ-Pyrone Dicarboxylic Acids

Meconic Acid and Chelidonic Acid

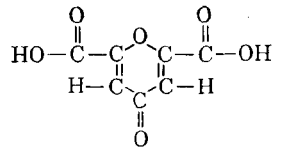
By SADAICHI MIYAMOTO† and EINAR BROCHMANN-HANSSEN

Meconic acid and chelidonic acid are very strong acids having dissociation constants of the same order of magnitude as sulfuric acid. This high acidity is explained on the basis of the resonance structure of the γ-pyrone molecule.

THE TWO MAIN acids of opium are meconic acid and sulfuric acid, which together account for 60 to 80% of the total acid content (1). In the course of our studies of the acid composition of opium it became apparent that meconic acid is an exceptionally strong acid, from the point of view of being an organic carboxylic acid. Search of the literature failed to reveal any information about the extent of its acid strength. This led to an investigation of the dissociation constants of meconic acid and the closely related chelidonic acid.



Meconic acid



Chelidonic acid

EXPERIMENTAL

Preparation of Meconic Acid.—Ten grams of powdered opium was mixed with 50 Gm. of Dowex 50-X2(H^+), 50–100 mesh, and 500 ml. of hot water in a 1 L. Erlenmeyer flask. The mixture was shaken mechanically for 30 minutes. The alkaloids, amino acids, and most of the colored materials were adsorbed on the resin, while the organic and inorganic acids were liberated. The supernatant liquid was decanted, 250 ml. of hot water was added to the resin, and the shaking was continued for 15 minutes. This process was repeated. The combined acid extracts were concentrated to about 100 ml. under reduced pressure, and powdered barium hydroxide was added until the solution was distinctly basic to litmus. The precipitate which formed was collected by centrifugation and washed several times by suspension in a little cold water, followed by centrifugation. It was treated repeatedly with small portions of 1 *N* hydrochloric acid to dissolve barium meconate, leaving a residue of barium sulfate. A test for complete removal of barium meconate was made by means of ferric chloride reagent (5% ferric chloride in 0.1 *N* hydrochloric acid). This reagent gives a red color with meconic acid. The combined extracts of barium meconate were neutralized with 10% ammonia, whereby barium meconate again precipitated out. The suspension was centrifuged, the precipitate washed several times with small amounts of cold water, and then transferred to a 250-ml. Erlenmeyer flask with the aid of about 100 ml. of water. Ten grams of Dowex 50-X_2 (H^+), 50–100 mesh, was added and the mixture was shaken mechanically for 30 minutes. The solution, containing free meconic acid, was filtered through a pledget of glass wool and the ion exchange resin was washed with water. The combined filtrate and washings were concentrated under reduced pressure at a temperature below 50°. Meconic acid crystallized and was recrystallized several times from water as the trihydrate. Titration with 0.02 *N* sodium hydroxide gave a purity of 99.8%. Anhydrous meconic acid was obtained by heating to constant weight at 102°. Upon titration the anhydrous sample showed a purity of 100 ± 0.2%.

Preparation of Chelidonic Acid.—Chelidonic acid was synthesized from ethyl oxalate and acetone according to the method described by Riegel and Zwilgmeyer (2). It was recrystallized from water as the monohydrate with a purity of 99.5%. Anhydrous chelidonic acid was obtained by heating at 160° to constant weight.

Determination of Dissociation Constants.—*Titration Method.*—Solutions of meconic acid and chelidonic acid (10^{-3} *M*) were titrated potentiometrically at 25° using a Beckman model W pH meter in combination with a Brown recording potentiometer, the scale of which was graduated in pH units. The titrant, 1 *N* sodium hydroxide, was added at a slow, constant rate by means of a synchronous clock motor driving the plunger of a small syringe. A polyethylene capillary tubing led from the syringe into the solution being titrated. The titration curves, which are illustrated in Fig. 1, show that both acids are very strong acids giving a sharp

Received April 10, 1961, from the University of California, Medical Center, School of Pharmacy, San Francisco.
Accepted for publication July 6, 1961.
This work was supported by a research grant (M3487) from the National Institutes of Health, Bethesda, Md.
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The authors are indebted to Dr. Calvin M. Lee who carried out the dipole moment measurements, and to Dr. W. D. Kumler for his helpful advice and suggestions regarding the interpretation of our data.

TABLE I.—DISSOCIATION CONSTANTS FOR MECONIC ACID AND CHELIDONIC ACID CALCULATED FROM THE NEUTRALIZATION CURVE AT 25°

Neutralization Equivalents of Alkali	$(Na^+) \times 10^3$	pH		pK	
		Meconic Acid	Chelidonic Acid	Meconic Acid	Chelidonic Acid
0.25	1.25	2.23	2.32	...	...
0.50	2.50	2.30	2.38	...	...
0.75	3.75	2.39	2.47	...	...
1.25	6.25	2.62	2.70	2.20	2.43
1.50	7.50	2.79	2.88	2.12	2.37
1.75	8.75	3.09	3.17	2.07	2.28
2.25	11.25	9.68	...	10.16	...
2.50	12.50	10.09	...	10.09	...
2.75	13.75	10.44	...	10.05	...

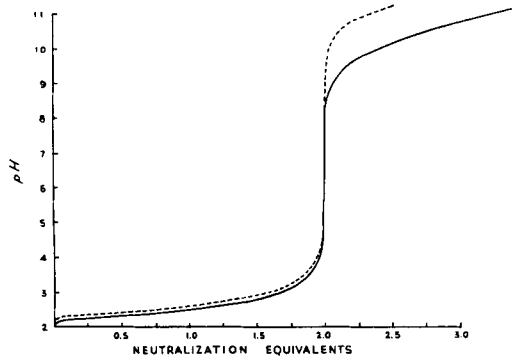


Fig. 1.—Titration curves for meconic acid (solid line) and chelidonic acid (broken line).

change in pH at the neutralization point. Chelidonic acid produces a single break after the addition of two equivalents of alkali, indicating that the two dissociation constants are relatively close. Meconic acid exhibits a second, but much less distinct potential change after addition of three equivalents of base.

The dissociation constants were calculated from the following equation

$$K_a = \frac{[H^+]\left[(Na^+) + (H^+) - \frac{Kw}{(H^+)}\right]}{M - \left[(Na^+) + (H^+) - \frac{Kw}{(H^+)}\right]}$$

where M is the total molar concentration of the acid. Calculations were made at the 0.25, 0.5, and 0.75 neutralization points (Table I). No value could be obtained for pK_1, since the calculations yielded negative results for this constant.

Spectrophotometric Method.—The results obtained by the titration method were checked by a spectrophotometric procedure (3). Meconic acid and chelidonic acid were found to obey Beer's law in the range of 230 to 315 mμ in both acidic, neutral, and basic solutions. Buffer solutions were prepared in range of pH 1 to 11.5. These were adjusted to constant ionic strength by means of sodium chloride and diluted with water to an ionic strength of 0.015 to 0.03 (Table II). Solutions of hydrochloric acid and sodium hydroxide in concentrations of 0.1 N and 1 N were also used for pH adjustment.

The determinations were carried out as follows: in a 25-ml. volumetric flask was placed 0.25 ml. of a solution of meconic acid or chelidonic acid, 5×10^{-3} M, and the volume was adjusted to the mark with buffer solution (or with hydrochloric acid or sodium hydroxide). Thus, a final concentration or 10^{-5} M was obtained. The final pH of the solution was determined with a Beckman model G pH meter and the absorbance was read in a Beckman DU spectrophotometer at 25°. The following wavelengths were chosen to give maximum change in absorbance with a change in pH; for meconic acid: 295, 305, and 315 mμ (Fig. 2); for chelidonic acid: 250, 260, and 270 mμ.

TABLE II.—BUFFER SOLUTIONS USED

pH Range	Composition	Ionic Strength
1.0–2.8	HCl + KCl	0.015
3.0–5.6	CH_3COOH + CH_3COONa	0.02
6.0–7.8	KH_2PO_4 + NaOH	0.02
8.0–11.47	H_3BO_3 + NaOH	0.03

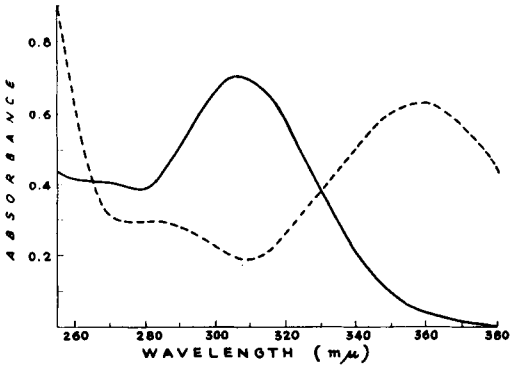


Fig. 2.—Ultraviolet spectrum of meconic acid in 0.1 N hydrochloric acid (solid line) and in 0.1 N sodium hydroxide (broken line).

The degree of ionization (α) was calculated from the following equation

$$\alpha = (k_\alpha - k_m)/(k_i - k_m)$$

where k_m = molar absorptivity of the unionized form, k_i = molar absorptivity of the completely ionized form, and k_α = molar absorptivity of the partly ionized form. Then, pK was calculated as follows

$$pK = pH - \log [(\alpha)/(1 - \alpha)]$$

In the region of low pH values there was very little change in absorbance with a change in pH. It was, therefore, not possible to differentiate

TABLE III.—DETERMINATION OF pK_3 OF MECONIC ACID BY SPECTROPHOTOMETRY AT 25°

pH	295 mμ A	295 mμ pK	305 mμ A	305 mμ pK	315 mμ A	315 mμ pK
7.17	0.411	...	0.443	...	0.366	...
7.70	0.411	...	0.443	...	0.366	...
7.99	0.409	10.133	0.442	9.528[a]	0.364	10.109
8.27	0.407	10.103	0.438	10.097	0.363	10.206
8.65	0.401	10.074	0.432	10.125	0.357	10.096
8.94	0.395	10.148	0.423	10.142	0.351	10.154
9.08	0.386	10.079	0.412	10.076	0.342	10.073
9.53	0.352	10.092	0.371	10.098	0.309	10.082
9.91	0.295	10.044	0.305	10.071	0.256	10.045
10.19	0.246	10.010	0.247	10.050	0.212	10.028
10.50	0.200	9.978	0.185	9.992	0.172	10.032
12.48	0.142	10.750[a]	0.110	10.433[a]	0.138	11.627[a]
14.00	...	...	...	...	...	...
(1 *N* NaOH)	0.137	...	0.105	...	0.106	...

[a] Extreme values not included in calculation of mean.

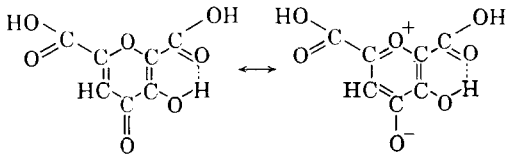
clearly between the molecular form and the ionized form on the basis of spectral changes for the first and second dissociation constants. Consequently, no dependable values could be obtained. The third dissociation constant for meconic acid was readily calculated and gave results that agreed closely with those obtained by the titration method (Table III).

Determination of Dipole Moment.—The dipole moment of anhydrous meconic acid was measured in dioxane at 30° (4). No measurement was made for chelidonic acid because it was not sufficiently soluble in dioxane or any other solvent suitable for dipole moment studies.

RESULTS AND DISCUSSION

The overall results are summarized in Table IV. The ionization constants reported are mean values based on Table I and Table III.

The results show that both meconic acid and chelidonic acid are very strong acids having dissociation constants very close to those given for sulfuric acid (5). This high acidity is probably due to the peculiar character of the γ-pyrone structure favoring a separation of charge by participation of the benzenoid form in a resonance hybrid



The positive charge on the ring oxygen will be expected to increase the acidity by the inductive effect. The high dipole moment for meconic acid also supports this hypothesis.

γ-Pyrone itself has a dipole moment of 4D (6). It has been calculated that if γ-pyrone were completely in the form with a separation of charge, its dipole moment would be 22D (6). Likewise, it has been estimated that its keto form would have a dipole moment of 1.75D (7). The actual situation is, therefore, a resonance hybrid to which both forms contribute.

TABLE IV.—DISSOCIATION CONSTANTS FOR MECONIC ACID AND CHELIDONIC ACID AND THE DIPOLE MOMENT OF MECONIC ACID

	Meconic Acid	Chelidonic Acid
pK_1	...	...
pK_2	2.13	2.36
pK_3	10.10[a] (10.08[b])	...
μ	5.19 D	...

[a] Titration method. [b] Spectrophotometric method.

The inductive effect of the hydroxyl group and hydrogen bonding between the hydroxyl group and the carboxyl group would tend to increase the acid strength of the latter. This would explain why meconic acid is a stronger acid than chelidonic acid.

Infrared spectra indicated the presence of hydrogen bonding in meconic acid, but were otherwise not very helpful for characterization of the structure.

Opium contains about 7 to 13% meconic acid and 2 to 4% sulfuric acid (1). The presence of such high concentrations of very strong acids is probably biologically significant in view of the high alkaloidal content of opium. In the same connection, it is interesting to note that *Chelidonium majus* L., in which the alkaloids also are localized in the latex, contains chelidonic acid, another strong dibasic acid.

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Histological Study of *Aristolochia triangularis*, *Casearia sylvestris*, and *Schinopsis balansae*

By PHILIP A. BABCOCK and MAYNARD W. QUIMBY

The content of this paper is concerned with the external and internal morphology of three little-known South American plant drugs: the dried stems of *Aristolochia triangularis* (*Aristolochiaceae*), the dried stem bark of *Casearia sylvestris* (*Flacourtiaceae*), and the dried leaves of *Schinopsis balansae* (*Anacardiaceae*). Stained and unstained sections were employed in this study, as were temporary and permanent mounts. The results of histological studies of powdered and macerated materials are also reported.

THERE is little information in the literature concerning the histology of *Aristolochia triangularis* Chamisso, of *Casearia sylvestris* Swartz, and of *Schinopsis balansae* Engler. Parts of these plants are reportedly used in a ground drug mixture employed by some South American Indians as a remedy for arthritic conditions. This problem was undertaken in order to enter macroscopic and microscopic descriptions of these crude drugs into the literature. The crude drug materials used in this problem were authenticated by Professor Heber W. Youngken.

The histologies of the stems of *Aristolochia triangularis*, the bark of *Casearia sylvestris*, and the leaves of *Schinopsis balansae* have been worked out and are described in this paper.

ARISTOLOCHIA TRIANGULARIS CHAMISSO

Description of the Plant.—*Aristolochia triangularis* Chamisso, family *Aristolochiaceae*, is a tropical woody vine indigenous to Brazil. Bailey (1) reports that it has been cultivated in California. There are about 300 species of *Aristolochia* found in the tropical and the temperate regions of the world. Most members of this genus are woody climbers that commonly possess irregular and grotesque flowers. These flowers often have an equally strange odor. According to Standley (2) the roots of a related species, *A. grandiflora* Swartz, have reportedly been used to poison human beings and animals in the West Indies and that they possess sudorific, abortive, and emmenagogue properties. He further reports that the roots of this and of several other species are used in preparing antidotes for snake bite.

The stems of *Aristolochia*, according to Schenke (3), are soft and tortuous because the ray parenchyma tissue is extensively developed. The soft phellem is thick and possesses numerous longitudinal fissures. Bailey (1) describes the plant as follows: the leaves are simple, deltoid, and glabrous, and have acute apices. They are pellucid-punctate and are palmately veined with three main veins. The lamina is averagely 4 inches long by 6 inches wide, and the petioles are prehensile. Each flower is borne singly and possesses a purple-spotted calyx. A corolla is absent. The fused sepals of the calyx form a bent tube with an oval, basal swelling. The termination of the tube is somewhat constricted, and at this point it widens out into a flat flare. The ovary of the flower is inferior with a lobed and fleshy style. The general floral shape is shown by the flowers of the common Dutchman's pipe, *A. macrophylla* Lam. The fruit is a 6-valued capsule.

Crude Drug.—The material used in this investigation was received as a bundle of dried stems, approximately 2 feet long by 8 inches in diameter. Pieces of stem varied in length from 1 foot to 4 feet and in diameter from 0.25 to 1 inch. The bundle had evidently been made from fresh material and was tied with a young stem. The ends of the stem segments were either cut diagonally or twisted off. The longer stems were bent double. The stem segments were very light in weight. The periderm is thick and very soft and possesses numerous deep longitudinal fissures. The bark is easily separated from the wood and is from tan to brown in color. The wood is light gray. There are occasional white patches of fungal growth on the periderm. The stems are rarely branched. The fracture is complete, brittle, and splintery. The severe drying that this material had been subjected to resulted in some breaking down of tissues and in considerable shrinkage.

Stem Histology.—In transverse section the arrangement of the vascular tissue is indicative of an abnormal woody dicotyledonous stem (Fig. 1). The periderm is layered and is fairly thick. Directly within the periderm lies a sclerenchymatous pericyclic zone made up of lignified cells arranged in an unbroken ring. The phloem consists of dome-shaped patches separated by large fan-shaped rays. The cambial zone is somewhat irregular, for it curves inward at the radii of the xylem rays. This results in a wavy general appearance. The xylem consists of wedge-shaped segments as well as broad rays, arranged in a ring. The xylem segments are usually seven in number and are penetrated by later-formed rays in the older stems. This vascular arrangement is known as an anomalous structure. The pith is small and irregular.

The phellem comprises the broadest area of the periderm. This region is layered with alternating light and dark colored layers. These layers are due

Received August 21, 1959, from the Division of Pharmacognosy and Allied Sciencies, Department of Biological Sciences, Massachusetts College of Pharmacy, Boston 15.

Accepted for publication August 10, 1961.

This paper is based on a portion of a thesis submitted by Philip A. Babcock to the Faculty of the Massachusetts College of Pharmacy in partial fulfillment of the requirements for the degree of Master of Science.

Presented to the Scientific Section, A.PH.A., Cincinnati meeting, August 1959.

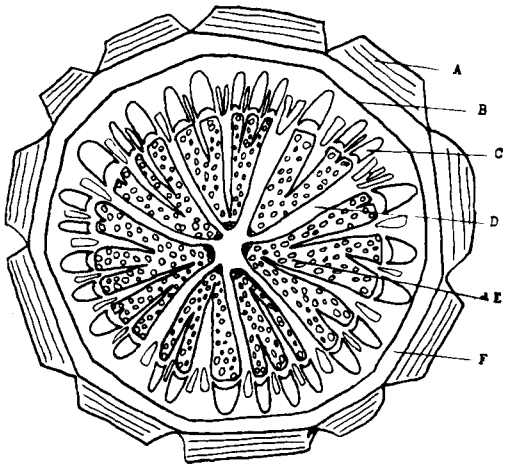


Fig. 1.—Transverse sectional diagram of *Aristolochia triangularis* stem. A, Phellem; B, pericycle; C, phloem; D, xylem ray; E, xylem "segment"; F, phelloderm.

to the variation in the radial dimensions of the cells. The light colored layers are composed of cells, each with a broad radial dimension. The more compact zone is composed of cells, each with a short radial dimension. These phellem layers are not uniform in width throughout. The phellem cells are laid down in radial rows and vary from large, nearly cubical cells to small cells that are nearly rectangular when viewed in transverse section. The cell walls are thin and often wavy. However, the outer walls of the cells are generally thicker than the radial and inner walls. The cells of the phellem contain a large amount of dark brown extractive matter.

The phellogen zone is fairly distinct and is characterized by somewhat compressed rectangular thin-walled cells arranged in radial rows. It is a narrow zone of only a few cells in width.

The cells of the phelloderm are arranged in radial rows and are tangentially elongated. These cells are relatively thin walled. Stone cells are also abundant in this region. These cells are scattered, or are found singly, or in clusters of from two to five cells. These stone cells are tangentially elongated and are characterized by having thick, lignified secondary cell walls with simple pits and branching pit canals. Secretory cells are found scattered throughout the phelloderm. These cells are three to four times larger and less elongated and possess walls that are thin but more sharply defined than the other parenchyma cells of this tissue. The secretory cells contain usually one globular, yellow-orange body, presumed to be oleoresinous.

The inner border of the periderm lies adjacent to an unbroken band of sclerenchyma cells averaging between five and eight cells in breadth. This pericyclic band exhibits two types of cells. That is, it is made up of a band of small, nearly isodiametric, compactly arranged stone cells that is often interrupted by patches of thin-walled fibers. When viewed longitudinally, the pericyclic fibers are seen often to possess septations. The thin walls of the fibers take only a light lignin stain, and have small, oval to slit-like, simple pits. The fibers are long and narrow, and have blunt, tapering ends. One average-sized septate fiber was found to be 1140 μ long with an average width of 27 μ. The stone cells of this region are small and possess thick, heavily lignified walls. Starch grains are rarely observed in the pericyclic sclerenchyma cells.

Stone cells are abundant in the phloem patches, but phloem fibers are absent. The stone cells are found in groups or are scattered. These stone cells possess heavily lignified thick walls. The stone cells in the phloem are fairly isodiametric with four to six sides and a somewhat rounded outline, except those adjacent to the cambial zone, where they are elongated. These are generally three to four times longer than they are wide, with their long axes in sections at right angles to the radius. These elongated stone cells are thinner walled than the scattered stone cells and they possess larger lumina. Furthermore, they are sharply rectangular in outline and are arranged in compact tangential rows, two to four cells in width. The cell walls exhibit numerous simple pits with unbranched pit canals.

Many secretory cells are present, scattered among the cells of the phloem. The secretory cells are large, thin walled, cubical cells with fairly regular outlines. They contain yellow-orange globular cell inclusions.

Phloem parenchyma cells and sieve elements are scattered, and they are indistinct in outline because of the extensive drying to which the material has been subjected. The large fan-shaped rays that separate the phloem patches contain numerous stone cells. These stone cells are thick walled and sharply rectangular in outline when examined in transverse section. The stone cells in the proximity of the cambial zone are more elongated and are arranged end to end in rows which follow the general contour of the phloem rays. The scattered stone cells of the phloem rays are usually situated with their long axes in a radial plane. The ray parenchyma cells contain starch grains more commonly than do the parenchyma cells of the phloem patches. Rarely, small druses are found in the phloem parenchyma.

The cambial zone dips inward between the xylem wedges, and this gives a scalloped appearance in transverse section to the wood region. The cambial zone is composed of a few rows of delicate compressed cells.

The fluted xylem, that is typical of *Aristolochia* and *Menispermum* and of some other vines, is due to abnormal secondary xylem formation. The xylem tissue of *A. triangularis* is divided into wedge-shaped segments, usually seven in number. These segments are often dichotomously divided by the formation of later formed rays. The number of later formed rays increases with the age of the stem. The xylem wedges of the young stems exhibit few of these later formed rays, whereas these rays become increasingly numerous in the older stems until the outer region of the xylem is composed of nearly equal, alternating areas of xylem wedges and rays. Growth rings are not evident.

Two general sizes of vessels are present. The more prominent size is up to 300 μ in diameter. The vessels are numerous and are distributed uniformly throughout the xylem segments. The wood is therefore ring-porous. The vessel members

(elements) here are as broad as or broader than they are high. Vessels with smaller diameters are more numerous. The members of these vessels are more elongated, being two to four times as high as they are wide. These members possess nearly transverse end walls that are characterized by large single pores. The side wall pitting is profuse. The pits are reduced and bordered, varying from large oval pits with distinct borders to small slit-like pits with indistinct borders. Tyloses are present in some vessels of the older regions of the xylem. Occasionally the lumina of some vessels are completely filled by the tyloses. The tyloses are commonly lignified and possess many simple pits that vary from small and slit-like to large and circular.

Full-bordered pit-pairs between vessels as well as between vessels and fibers appear in face view as circles, each enclosing a cross. A longitudinal section showing a vessel wall junction reveals that the pits have small lenticular pit cavities and broad pit canals leading into the cell lumina. These are somewhat similar to the bordered pits of the conifers but they are much reduced.

In transverse view the numerous fibers appear angular in outline, while in longitudinal view they are usually long and tortuous, and they possess characteristically tapering ends. For the most part these fibers have walls that are approximately one-sixth as thick as the cell is broad. This was determined from examination of macerated material. These narrow fibers are, on the average, 1 mm. long. On their side walls they possess many slit-like pits with indistinct borders.

The presence of primary xylem is evidenced by the presence of vessels with annulo-spiral wall thickenings. The primary xylem elements are found on the inner extremities of the xylem wedges adjoining the pith area.

Sparsely scattered wood parenchyma cells occur both singly and in small clusters. These cells are relatively large. They are elongated up to eight or ten times their width. In macerated preparations they often show a distorted outline. The walls of these cells are thickened and lignified. Numerous round or oval simple pits occur in the walls. Even on the same wall these pits vary considerably in size. Small clusters as well as single, very small starch grains are commonly found within these cells.

According to Schenke (3) there are eight initial xylem rays extending from the cambium to the pith. In this investigation seven rays were found to be the more common number. The formation of younger rays divides the initial xylem segments as the stem increases in age. Many older stems show equal, alternating areas of ray and xylem wedge areas in the proximity of the cambial zone.

Ray parenchyma cells, secretory cells, and stone cells are present in the xylem rays. The parenchyma cells are thin walled and rectangular in longitudinal section. In transverse section they appear slightly elongated radially. Stone cells are usually found scattered through the deeper third of some of the rays near the pith. These stone cells are nearly isodiametric and most commonly occur singly, although occasional clusters of two or three are present.

The secretory cells found in the xylem rays are much larger than the ray parenchyma cells. They possess thin but clearly defined walls. Most of them contain a globular, yellow-orange body. These secretory cells are scattered and are not so numerous as the ray parenchyma cells, and they are generally larger but less elongated.

Starch grains are more abundant within the ray parenchyma cells than within any other cells of the xylem.

Crystals in the form of small druses and, rarely, prisms are often present as cell inclusions in the xylem ray cells. Druses of very fine crystals and irregular prisms are not commonly situated within the parenchyma cells bordering the fibers of the xylem. Commonly, only one crystal is present within a single cell, but near the pith region more are sometimes present.

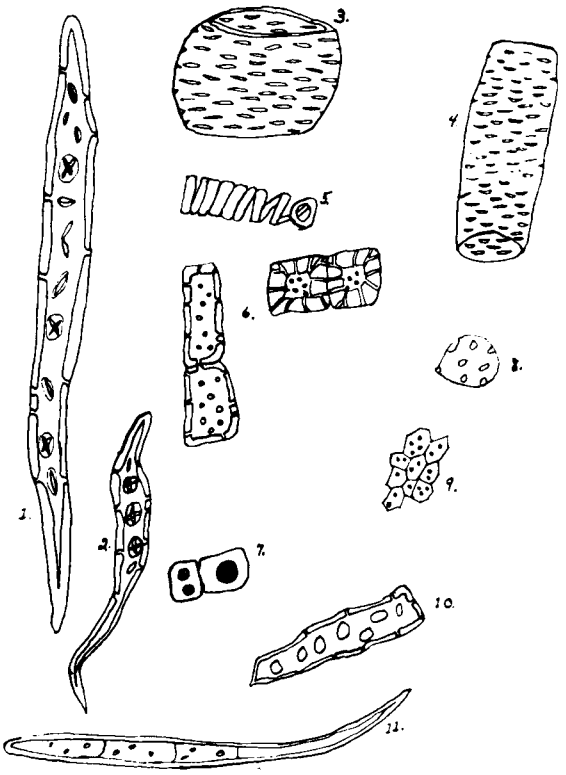


Fig. 2.—Elements of macerated and powdered *A. triangularis* stem. 1 and 2, Wood fibers; 3 and 4, vessel elements; 5, spiral element; 6, stone cells; 7 secretory cells; 8, tyloses; 9, periderm cells; 10, wood parenchyma; 11, septate pericyclic fiber.

The pith area is relatively small and it is irregular because the inner portions of the xylem wedges are not equidistant from the center of the stem. It is composed of typical thin-walled parenchyma cells with coarse simple pits. The cells are loosely arranged. Stone cells occur both singly and scattered in small clusters. They are fairly isodiametric, thick walled, and heavily lignified. Crystals occur more abundantly in the pith than in other tissues of the stem. These crystals are coarser than those of other tissues. They are prismatic or diamond-shaped, and are thin.

Powdered and Macerated Material.—Stem seg-

ments were broken and then ground in a drug mill. The resulting powder was screened through a 40-mesh screen. This resulted in a homogeneous brown powder with no odor.

Other portions of stem material were treated according to the Schulze maceration process. Stem segments were cut into thin longitudinal slices and were put into a beaker. A mixture of one part nitric acid and three parts water was added, and this was followed by a few crystals of potassium chlorate. The beaker was then warmed until bubbles and yellow fumes arose from the liquid. The reaction was allowed to continue until the pieces of wood became white. The material was then removed, washed with running water, and shaken with glass beads to dissociate the cellular elements. Microscopic examination of the powdered and macerated materials revealed the following (Fig. 2): in mounts of the powder, fragments of xylem are prominent. These are composed mostly of clusters of wood fibers and some small vessels. Fragments from the walls of the larger vessels are fairly numerous. Occasional tyloses lignified with numerous large simple pits are present. These have been separated from the walls of the older, large vessels. Fragments of periderm are easily detected because of the characteristic thin and wavy-walled cells of the phellem. Fragments of parenchyma tissue from the rays, pith, and phloem are present. Secretory cells are often present in the parenchymatous tissue fragments. There is usually only one yellow-orange body within any single secretory cell. Occasionally these cell inclusions are found separated from the secretory cells. Stone cells are either associated with parenchyma tissue or they are discrete. There are two general types of stone cells. One type is nearly isodiametric and thick-walled. The other type is elongated. A few small starch grains are present which vary from 3 to 5 μ in diameter. They are angular and possess a faint oval eccentric hilum. Occasional fragments as well as whole crystals are present as rectangular and diamond-shaped platelets and prisms.

Large barrel-shaped vessel elements are readily seen in macerated material. They show numerous bordered pits on their side walls. The pits vary from small slits to large ovals, within rounded borders. The end walls are nearly transverse, each with a single large pore. Many of these large vessels measure up to 300 μ in diameter and are sometimes broader than they are high. One vessel element was found to be 340 μ broad by 290 μ high. The smaller vessels are not nearly so broad, and they are commonly two to four times higher than they are broad. Xylem fibers are thick-walled and are, on the average, 750 μ long by 25 μ wide. They are commonly tortuous and possess numerous slit-like bordered pits. Less common are the thin-walled septate fibers of the pericycle. These average a little over 1000 μ in length by 25 to 30 μ in width. They have small, rounded, simple pits on their thick walls. Stone cells are numerous. An elongated stone cell of average size measures 100 by 36 μ. The other type of stone cell is more nearly isodiametric, thicker-walled, and possesses branching pit canals. The lumen is much smaller in the latter type. Small segments of phellem tissue are present, as are some thin-walled parenchyma cells.

CASEARIA SYLVESTRIS SWARTZ

Description of the Plant.—*Casearia sylvestris* Swartz, family *Flacourtiaceae*, occurs as a shrub or tree that ranges from 3 to 20 meters in height. It is indigenous to the West Indies and to Central and South America. The genera *Hydnocarpus* and *Taraktogenos*, some species of which yield chaulmoogra oil, are also members of this family.

According to Standley (4) the branches of this tree are slender and bear simple alternate leaves that are usually entire. The leaves are glabrous or nearly so with lanceolate to lanceolate-oblong outlines and acute apices. They are from 6 to 10 cm. in length by 2 to 3 cm. in width, and they are thin and densely pellucid-punctate.

Small greenish-white flowers of this plant are borne in umbels on pedicels which are 2 to 4 cm. The fruit is small and obovoid and is 3 to 4 cm. in length.

The pale yellow wood of the tree is hard, heavy, and compact. The tree is used as a source of lumber in Cuba.

Many common names have been given to this plant in the countries of its origin. Some of these are as follows: *comido de culebra*, Nicaragua; *cafeillo cimarron* and *laurel espada*, Puerto Rico; *sarne de perro*, Cuba and Puerto Rico; *rompe-hueso* and *sarnilla*, Cuba.

Crude Drug.—The part used is the stem bark which occurs as curved chips up to 6 inches long. Usually the pieces are not nearly so wide as they are long and they are, on the average, $^1/_4$ inch in thickness. The convex outer surface is brownish-tan with a scaly appearance caused by the sloughing off of small platelets of periderm. Small white areas of fungal growth and some adherent moss and lichens are sometimes evident. The fracture is complete, short, and sharp. The broken transverse surface shows narrow alternating tangential layers of light-brown sclerenchyma tissue and dark brown nonlignified tissue. The concave inner surface is grayish-tan. It is smooth except for slight longitudinal striations. Small white areas of fungal growth are rarely present on this surface. The odor is slightly musty.

Histological Data.—Beneath a thin layer of periderm are alternating strata of nonlignified and of lignified cells. There are numerous, but narrow, phloem rays present. Because of the nature of this material, its histology is essentially that of secondary phloem. The amount of periderm tissue present is usually slight (see Fig. 3).

The phellem is $^1/_8$ inch thick, on the average, and varies from 6 to 20 cells in thickness. The inner and radial walls of these cells are thickened and lignified. They are narrow, tangentially elongated cells with small lumina and are arranged in definite radial rows. The phellogen is indistinct and no phelloderm can be demonstrated. The later formed periderms arise in the outer regions of the secondary phloem, and this results in the sloughing off of the outer tissue in some areas.

The lignified layers of the phloem are made up predominately of stone cells that are elongated vertically. These cells are one and one-half times as high as broad. Tangential bands of stone cells course through the phloem in a regular manner with only occasional dividing or joining. Because the

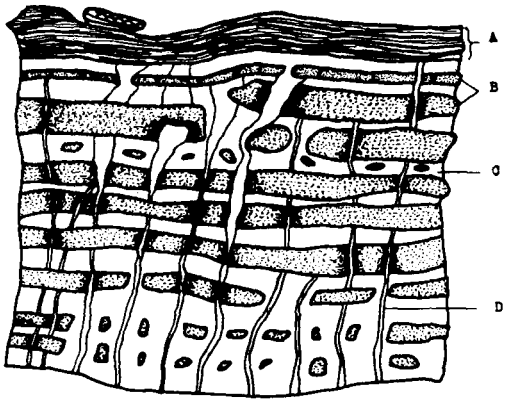


Fig. 3.—Transverse sectional diagram of *Casearia sylvestris* bark. A, Periderm; B, stone cell strata; C, parenchymatous stratum; D, phloem ray.

cells of these areas are very similar in size and shape, they form a regular pattern of rows both tangentially and radially. This pattern is referred to as a "storied arrangement." In transverse section the stone cells of these bands possess fairly regular outlines and have thickened, heavily lignified walls. The walls are densely pitted with simple pits and have unbranched pit canals. The majority of these stone cells contain a single large crystal that often completely fills the cell lumen. The strata of stone cells vary from 2 to 25 cells in radial dimension. The strata are continuous in the inner region of the phloem, but they become increasingly discontinuous in the regions nearer the periderm. These discontinuous strata of lignified cells are interrupted by phloem ray cells and by other cells of the nonlignified strata. Included in the lignified strata are also definite aggregations of nearly isodiametric stone cells. These stone cells are thicker-walled than the other stone cells of the strata and they possess branching pit canals but they rarely contain crystals. Similar stone cells are occasionally found singly or in small clusters among the cells of the nonlignified strata. These cells are infrequently found in the lignified strata. These cells are five to eight times longer than wide, with walls about $^1/_6$ as thick as the cell is wide. Each possesses a broad lumen. One end wall is often oblique, and the other end wall is at a right angle to the longitudinal walls. All the walls of these cells possess numerous simple pits with branched pit canals.

The outermost stratum of lignified cells is made up of thinner-walled stone cells with larger lumina and fewer crystals than the stone cells of the inner strata.

The strata of nonlignified cells are composed of phloem parenchyma, sieve elements, ray parenchyma, and sparsely scattered stone cells. The sieve tube elements are thin-walled and possess oblique end walls. Their sieve plates are compound as seen in a radial longitudinal view. The phloem parenchyma cells are numerous. In longitudinal section these cells are seen to be from two to three times as high as they are broad. In transverse view these cells appear nearly square in outline.

The phloem rays are very numerous and their regular arrangement is often interfered with by the stone cell strata. Rays vary from two to five cells in width at an expanded middle portion, and they commonly possess a many-celled elongation on the top and on the bottom that is evident in tangential sections. These elongations of the rays are one cell in width. The ray cells vary in size, and each exhibits a rounded outline in a tangential view.

Starch grains occur in small clusters within some of the phloem parenchyma cells and rarely within the ray parenchyma cells. They are two- to four-compound.

Powdered and Macerated Material.—The chips of bark were ground in a drug mill and the powder was then put through a number 40-mesh sieve. The resulting powder was homogeneous and light tan in color, with no noticeable odor. Material prepared by the Schulze maceration process was also examined. The following elements were observed during a microscopic examination (see Fig. 4).

The most prevalent element present is the crystals. These crystals are greatly varied in shape and give a play of colors with polarized light. Some of the crystals still remain within the stone cells. Stone cells are very numerous. These occur singly and in clusters. The clusters are most commonly composed of one or more rows of cells. Solitary crystals are usually present in the lumina of the stone cells in these rows. Two types of stone cells are present. Thick-walled nearly isodiametric stone cells are present. They possess branching pit canals and

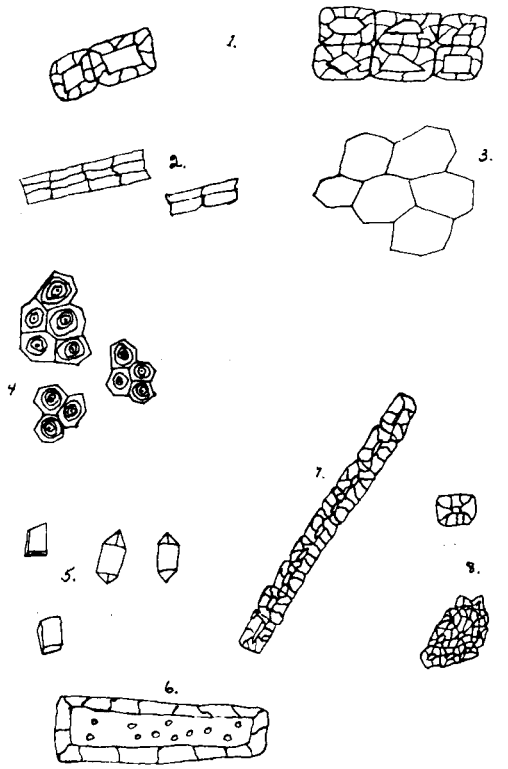


Fig. 4.—Elements of macerated and powdered *C. sylvestris* bark. 1, Crystal-bearing stone cells; 2 and 3, periderm fragments; 4, starch; 5, crystals; 6, elongated stone cells; 7, row of stone cells (crystals lacking); 8, stone cell cluster.

small lumina. These are, on the average, 30 μ in diameter. The thinner-walled stone cells are elongated. They average from 50 to 60 μ in length by 25 to 30 μ in width. These cells have unbranched pit canals and many simple pits. Their lumina are often angular because of the crystals that completely fill them. Clusters of phloem parenchyma cells and sieve elements are also found. Fragments of phellem are composed of narrow, tangentially elongated cells of about 10 by 30 μ. Phellem fragments in surface view appear as clusters of three- to six-sided cells. In macerated preparations they appear rounded, with clear, refractive cell walls and nonrefractive lumina. Small angular starch grains are present in small numbers. They occur commonly as three- to many-compound grains. The individual grains are, on the average, 3 to 4 μ in diameter. They possess tiny, faint, central hila and concentric lamellae.

SCHINOPSIS BALANSAE ENGLER

Description of the Plant.—*Schinopsis balansae* Engler is an anacardiaceous tree attaining a height of from 10 to 20 meters, indigenous, according to Cabrera (5), to the hot, dry forests of the southern Matto Grosso in Paraguay and northeastern Argentina.

Engler described the plant in 1885 (6). The bark of the trunk is ash gray in color and the wood is dark red and very hard. The young branches are light gray and sometimes possess sharp spines that are stout and straight, and from 1 to 2 cm. long. Simple leaves are borne alternately, and the twigs have short internodes. Each leaf possesses a semicylindrical petiole varying from 4 to 10 cm. in length. They are coriaceous, glabrous, and oblong to oblong-lanceolate. Their apices are very short and mucronate. The margins are entire and somewhat undulate, and the obtuse bases are rounded. The midrib is reddish, thick, and hard. There are numerous thin, but prominent, lateral veins. The leaf is from 4 to 8 cm. long by 1.5 to 2 cm. wide. The flowers are borne in terminal, pyramidal panicles having a length of 6 to 7 cm. Bractioles of the panicles are concave and ciliate, and about 1 mm. long. The pedicels are slightly pubescent. The fruit is a lustrous and woody single samara. It is knife-shaped and possesses an ovoid "seed body."

Leaves of this plant contain a considerable amount of a catechol-type tannin that is water soluble and that gives a dark green color with ferric chloride solution.

According to Youngken (7), the dried heartwood of *Schinopsis balansae* Engler and *S. lorentzii* (Grisebach) Engler are the sources of *Quebracho Colorado* which has been valued for its high tannin content. The name *Quebracho* means "axe-breaker." Therefore, the name *Quebracho Colorado* means "red axe-breaker wood." *Quebracho Colorado* is not related to *Quebracho blanco* which refers to the bark of *Aspidosperma quebracho-blanco* Schlectendal, family *Apocynaceae*. Preparations of the last named bark were once used as respiratory stimulants.

Cabrera (5) reports that the natural range of *S. lorentzii* borders on the north of that of *S. balansae*. The simple leaves of *S. balansae* enable one to distinguish this species easily from *S. lorentzii*, which possesses imparipinnate leaves, each with 7 to 15 leaflets.

Histological Data.—As seen in transverse section, the arrangement of tissues of the lamina is indicative of a bifacial leaf. The midrib area is somewhat semicircular in this view, the dorsal side of the midrib being more prominent than the ventral side (see Fig. 5).

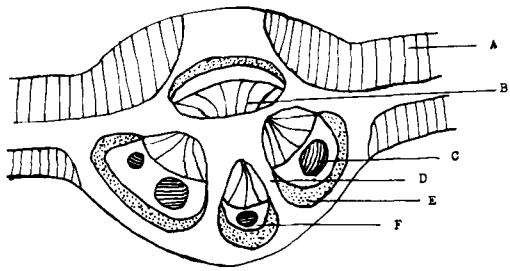


Fig. 5.—Transverse sectional diagram through the midrib of *Schinopsis balansae* leaf. A, Palisade zone; B, xylem; C, secretory canal; D, parenchyma; E, sclerenchyma fibers; F, phloem.

The upper epidermis of this leaf consists of a single layer of cells which are rectangular in outline. Their outer walls and the outer third of their radial walls are considerably thickened. In many instances these epidermal cells have lignified walls. There is a very well developed cuticle present. Stomates are present in about the same number as in the lower epidermis. The neighboring cells in this view appear somewhat L-shaped, with the base of the L forming a flange upon which the guard cells are situated.

Palisade cells are oriented with their long axes perpendicular to the epidermis. The palisade cell regions are each composed of from two to three layers. The palisade zones constitute three-fourths of the mesophyll of the lamina. The palisade regions are separated by a narrow region of fairly compact spongy mesophyll cells.

The lateral veins possess well developed bundle sheaths. The sheaths of the small veins are parenchymatous. Those of the larger veins are sclerenchymatous and they extend to both epidermises. A small group of fibers whose walls are thick and slightly lignified is usually associated with the phloem of the bundle. A single secretory canal is commonly present within the phloem of these large veins. Prismatic or diamond shaped solitary crystals are found in many of the cells of the bundle sheath. Characteristically large, coarse druses are sometimes present within the bundle sheath cells of the small veins.

The cells of the lower epidermis are very similar to those of the upper epidermis.

The epidermal cells of the midrib are about one-third the size of those on the rest of the leaf blade, but they are of similar shape. The palisade region within the upper epidermis extends into the midrib from each side to a distance of about one-third of the width of the midrib region. Beneath the upper epidermis of the midrib and between the palisade cell regions is an area of well developed collenchyma tissue. The most striking characteristic of the histology of this leaf is the arrangement of the

vascular tissue in the midrib. It is arranged in a broken semicircle of five vascular strands. Two inverted strands with the phloem regions toward the upper epidermis are situated ventrally, the other three strands are oriented with their phloem regions toward the lower epidermis. These strands form an arc under the upper strands. The secondary development of the xylem is fairly extensive, whereas the phloem region is not as well developed. The cambium of the bundles is indistinct.

Secretory canals are present within the phloem regions of the three lower vascular strands of the midrib and rarely within those of the upper vascular strands. From two to three canals occur within the two lateral strands, and one canal is found in the lowest strand. The content of these canals is presumed to be oleoresinous (see Fig. 5).

Transverse sections through the midrib at the base of the lamina show five vascular strands. Similar sections cut close to the apex show but one strand.

The center of the midrib is composed of parenchyma cells with thickened lignified walls that are marked by round simple pits. Narrow zones of parenchyma cells radiate out between the vascular strands and encircle the stele. Between this parenchymatous region and the lower epidermis is an extensive area of collenchyma. Many of these cells contain solitary crystals that vary from diamond-shaped to prismatic.

A region of thick-walled fibers is associated with the phloem region of each vascular strand. Commonly only the outer walls of these cells are lignified.

In surface sections of the lamina the epidermal cells present a fairly regular outline although the cell walls are irregularly thickened. Patches or small areas exhibit epidermal cells with lignified walls. These areas have no definite pattern. The lignified walls of these cells possess simple pits. Stomates occur about equally in each of the two epidermises. The guard cells of the stomates are surrounded by narrow neighboring cells. The number of neighboring cells is seven, on the average, but varies from a minimum of five to a maximum of nine. There are usually two neighboring cells along each side of the paired guard cells, two neighboring cells at one end of the pair, and only one at the other end. Rows of crystal-bearing cells accompany most of the veins. Such rows are parallel to the veins with which they are associated. The crystals are commonly diamond shaped or prismatic. Occasional one-celled, appressed, nonglandular hairs are associated with the epidermal cells of the midrib and of the larger lateral veins. These hairs are thick-walled and possess narrow lumina that extend about one-half the length of the hairs. The bases are only slightly enlarged. The hairs vary from about 35 to 90 μ in length. The average length of these hairs is approximately 60 μ and their bases are about 13 μ wide.

In transverse sections of the petiole one can see five leaf traces entering the base of the petiole. These traces are arranged in the semicircular pattern (see Fig. 6). There are three traces in an arc and their phloem regions face the lower epidermis. There are two small ventral traces across the top of the arc, and they are inverted so that their phloem regions face the upper epidermis. The xylem regions of the traces are separated by regions of

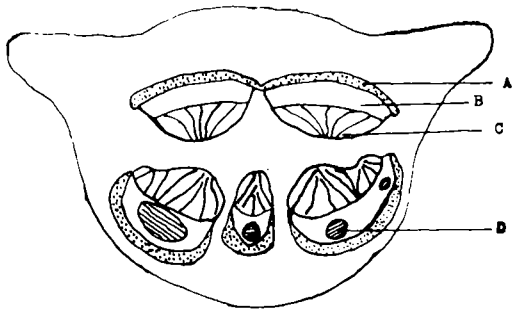


Fig. 6.—Transverse sectional diagram of *S. balansae* petiole. A, Sclerenchyma fibers; B, phloem; C, xylem; D, secretory canal.

parenchyma cells. The central portion of the midrib is composed of parenchyma cells whose walls are thickened and lignified. These cells are relatively large and have conspicuous intercellular spaces. In the phloem region of each of the three dorsal traces one or more secretory canals are situated. Each of the five traces possesses a definite sclerenchymatous cap of thick-walled fibers. The walls of these fibers seldom are lignified. Collenchyma is extensively developed between the vascular tissue and the epidermises. Many of these collenchyma cells contain crystals of the types found in the midrib. Crystals are sometimes found also within

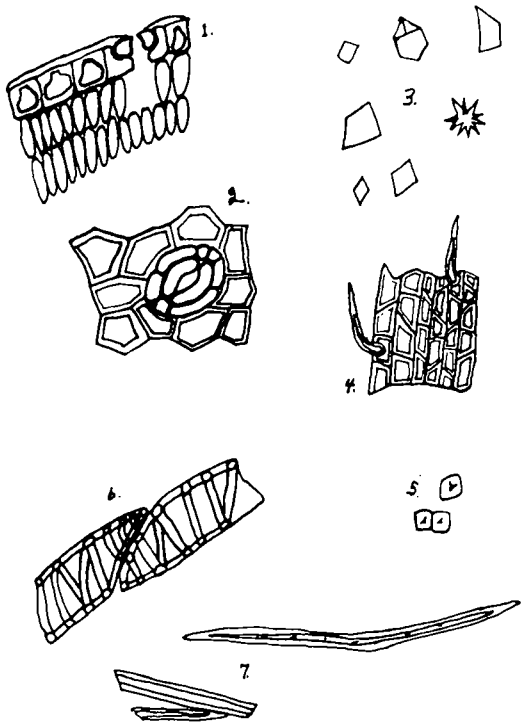


Fig. 7.—Elements of macerated and powdered *S. balansae* leaf. 1, Epidermal fragment with stomate and attached palisade cells; 2, epidermal fragment in surface view; 3, crystals; 4, hairs on epidermal fragment of petiole or midrib; 5, starch grains; 6, xylem elements; 7, sclerenchyma fibers.

the cells of the phloem regions and within the parenchyma cells in the center of the midrib.

Some slightly developed periderm can be found to have replaced the epidermis in scattered areas on the dorsal surface of the petiole base. It is formed from the subepidermal layer. Nonglandular hairs of the type found on the midrib are occasionally present on the dorsal surface of the petiole. The petiole has a normal clasping base. A short distance from the base the petiole becomes semicylindrical with the ventral side flattened. Its firmness is due to the extensive development of collenchyma tissue.

Powdered Material.—A sample of dried leaves was crumbled and then was screened through a 40-mesh sieve. The resulting powder was greenish and possessed a very slight musty odor. The following elements were observed in microscopic examination (Fig. 7): fragments of epidermis from the lamina are prevalent. The epidermal cells are polygonal and possess thick walls when seen in surface view. Occasionally some of the walls of the epidermal cells are lignified. Stomates are prominent in these epidermal fragments. The guard cells are long and elliptical. The number of neighboring cells varies from five to nine but is, on the average, seven. There are usually two neighboring cells along each side of the paired guard cells, two neighboring cells at one end of the pair, and only one at the other end. Fragments of mesophyll tissue are commonly associated with epidermal tissue.

Fragments of epidermis from the midrib and the petiole are composed of cells that are smaller than those found on the lamina, and these are arranged in rows with their end walls sometimes oblique when seen in a surface view. These epidermal cells have thick, but nonlignified, walls. No stomates are present in this tissue. One-celled, appressed, nonglandular hairs are often present on these fragments of epidermal tissue.

Broken clusters of thick-walled fibers with sparse, small, simple pits are present. Sometimes crystal-bearing cells accompany these fibers.

Fragments of xylem elements show spiral wall thickenings or scalariform pitting.

Occasional tissue fragments contain portions of the secretory canals with an amorphous red-brown content. Fragments of collenchyma tissue made up of cells with characteristic unevenly thickened walls are present. These cells are elongated. Crystals are a prominent element. They occur as fragments and as entire prisms, rarely as druses. The prisms are on the average 15 μ long. Starch grains are not common, but some do occur as simple or two-compound grains with diameters of from 4 to 5 μ.

SUMMARY

This paper contains reports on the histologies of the stem of *Aristolochia triangularis* Chamisso, the bark of *Casearia sylvestris* Swartz, and the leaf of *Schinopsis balansae* Engler. The following characteristics are noteworthy.

1. *Aristolochia triangularis* stem. This stem exhibits an anomalous vascular structure. The wood is diffuse porous. Wood parenchyma is sparse and diffuse. Thick-walled wood fibers are abundant. Stone cells occur scattered in the broad xylem rays. Some of the vessels contain tyloses. Some of the ray and pith cells contain prismatic and thin diamond-shaped crystals. The fan-shaped phloem rays consist, in part, of stone cells. The pericycle consists of stone cells and fibers. The extensively developed phellem is deeply fissured.

2. *Casearia sylvestris* bark. This bark consists mainly of secondary phloem, only a small amount of periderm being present. In the phloem numerous tangential bands of stone cells alternate with bands of nonlignified cells. Most of the stone cells contain crystals. The crystals are prismatic, nearly rectangular, or diamond-shaped. Sieve tube elements possess compound sieve plates on their radial walls. The small starch grains are usually two- to many-compound. Two types of stone cells are present. No fibers are present.

3. *Schinopsis balansae* leaf. This leaf possesses a well developed cuticle and the stomates are rather evenly distributed in the two epidermises. Stomates most commonly have seven neighboring cells. In scattered areas of the epidermis the cell walls are lignified. The lamina exhibits a bifacial structure. The midrib exhibits five vascular strands arranged in a semicircle. The two upper strands are inverted. The phloem areas of many vascular strands possess secretory canals. Fibers that accompany vascular strands are often nonlignified. Coarse druses and prismatic crystals are often associated with the vascular strands. Appressed nonglandular hairs are sometimes present on the dorsal surface of the petiole.

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Bioassay of Estradiol 17-Cyclopentylpropionate

By W. F. BEYER, D. H. EMMONS, and M. L. PABST

The immature mouse uterus has been utilized for the bioassay of a long-acting estrogen, estradiol 17-cyclopentylpropionate. The compound was dissolved in cottonseed oil and injected in a single subcutaneous dose of 0.10 ml. The uteri were removed and weighed 72 hours after the injection. Statistical analysis of uterine weights, using a 2 × 2 balanced bioassay, gave an approximate average standard error of potency of 12%. Similar uterine weights were produced by estradiol 17-cyclopentylpropionate when given in a single subcutaneous injection or when given in divided doses over a 3-day period. In the case of estrone and estradiol, a single dose was considerably less effective than divided daily doses. The similarity in response to one and three doses of estradiol 17-cyclopentylpropionate and its ability to maintain an increase in uterine weight following a single dose has been utilized as the basis of the assay for a long-acting estrogen.

RUBIN, *et al.* (1), reported a bioassay for estrogens using the increase in uterine weight of immature mice as the response. This method involved the use of a divided dose schedule and uterine ratios (100 times the uterine weight in mg. divided by the body weight in Gm.) as the response. Modifications of the method resulted in a shorter assay procedure for estradiol 17-cyclopentylpropionate (ECP).[1] The modified method is described for a single dose schedule and the use of uterine weights of immature mice. Uterine responses to estrone U.S.P. and estradiol U.S.P. are presented along with dose response curves and assay results for ECP.

ANIMALS AND METHOD

Twenty-one to twenty-five-day old immature mice of the CFW or CF No. 1 strain were used. No significant differences were noted in responses of the two strains. All mice utilized for a single test were of the same age group and strain. Commercial mouse diet and tap water were permitted *ad libitum*.

A cottonseed oil solution of ECP was injected subcutaneously in a single dose of 0.10 ml. The mice were sacrificed and the uteri were removed 72 hours after the injection. The uteri were separated from the ovaries, severed at the uterocervical junction, stripped of extraneous tissue, blotted free of contained fluid, and rapidly weighed to the nearest 0.10 mg. In many cases the body weights were obtained at the time the animals were sacrificed. This made it possible to obtain uterine ratios as well as uterine weights.

The log dose-uterine response relationship with a constant ratio of two between successive doses was used to calculate potency. The 2 × 2 assay design of Bliss (2) or U.S.P. XVI (3) was used. When more than one sample was assayed against a single standard, calculations for the joint assay of several preparations (4) were used.

RESULTS AND DISCUSSION

It was necessary to obtain dose response data and to test the duration of response before establishing an assay procedure. The duration of response was tested using both single and multiple dose schedules. By repeated trials it was found that a total dose of 0.32 mcg. of ECP per mouse resulted in a useful uterine weight response. For the single dose schedule, 0.32 mcg. of ECP was contained in a volume of 0.10 ml. of cottonseed oil. For the multiple dose schedule, 0.32 mcg. of the compound was contained in 0.30 ml. of cottonseed oil and 0.10 ml. was administered daily for 3 days. The uteri were removed at varying intervals after the injections to determine the duration of response.

The results in Table I show that the largest uterine weights and ratios occurred during the 48 to 120-hour period after a single injection and approximately 72 to 144 hours after the initial injection using multiple doses. Although the uterine weights remained fairly constant over most of the test period for both dosage schedules, the uterine ratio values decreased as the body weights of the mice increased. The body weights increased as the interval between initial injection and uterine removal was lengthened.

Dose response data for the single and multiple dose schedules were obtained using an interval of 72 hours from beginning of injection to uterine removal. At this time, uterine weights of mice receiving a total dose of 0.32 mcg. per mouse (Table I) were near maximal and were approximately five times that of uninjected controls (Table II). This period was also the earliest time for uterine removal when an injection interval of 24 hours was maintained for the multiple dose schedule.

The mean uterine weights and uterine ratios for the dosage levels tested are shown in Table II. The responses over a dose range of 0.08 to 0.64 mcg. of ECP per mouse exhibited a graded dose response relationship. There was a significant difference between the average responses for successive dosage levels tested, with the dose range suitable for bioassay extending over an eightfold difference in dose.

The data used in Table II were analyzed by linear regression methods yielding slopes (b),

Received November 8, 1960, from the Biological Control Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication September 25, 1961.
The authors acknowledge with appreciation the advice of Dr. O. S. Carpenter, Sc. Admn. Staff, The Upjohn Co., on statistical treatments. The assistance of R. J. Cole, Biological Control, The Upjohn Co., for computations using the IBM-610 computer is also gratefully acknowledged.

[1] Estradiol 17-cyclopentylpropionate (ECP)—The Upjohn Co.

TABLE I.—COMPARISON OF MOUSE UTERINE RESPONSE TO 0.32 MCG. ECP GIVEN IN A SINGLE DOSE OR IN THREE SUBCUTANEOUS DOSES[a]

	Hours after Beginning of Injection						
	24	48	72	96	120	144	168
One Dose							
Mean uterine weight (mg. ± S.E.)	21.6 ± 1.4	50.3 ± 3.3	45.2 ± 1.6	39.7 ± 1.7	48.0 ± 2.8	37.1 ± 2.5	...
Mean uterine ratio (± S.E.)	154 ± 8	329 ± 16	335 ± 10	271 ± 12	321 ± 19	240 ± 12	...
Mean body weight (Gm.)	14.0	15.2	13.6	14.9	15.1	15.5	...
No. of Mice	8	8	18	18	9	10	...
Three Doses							
Mean uterine weight (mg. ± S.E.)	...	...	47.5 ± 2.6	51.9 ± 2.7	44.7 ± 2.0	46.5 ± 2.6	40.7 ± 2.0
Mean uterine ratio (± S.E.)	...	...	321 ± 20	342 ± 18	279 ± 18	279 ± 17	230 ± 11
Mean body weight (Gm.)	...	...	14.9	15.2	16.1	16.8	17.8
No. of Mice	...	...	10	10	10	10	11

[a] Uteri removed at varying intervals after initial injection.

TABLE II.—COMPARISON OF UTERINE RESPONSE TO ECP GIVEN IN A SINGLE OR IN THREE SUBCUTANEOUS INJECTIONS[a]

Total Dose, mcg.	No. Mice	Mean Uterine Weight, mg. ± S.E.	Mean Uterine Ratio, ±S.E.
One Dose			
0.00	6	9.8 ± 1.1	77 ± 7
0.08	9	18.6 ± 1.6	143 ± 13
0.16	9	22.8 ± 2.7	193 ± 23
0.32	9	41.9 ± 4.0	349 ± 33
0.64	9	50.2 ± 5.1	367 ± 28
Three Doses			
0.00	7	9.5 ± 1.3	69 ± 7
0.08	14	16.3 ± 2.2	117 ± 16
0.16	14	33.0 ± 3.9	240 ± 27
0.32	14	46.1 ± 3.1	318 ± 21
0.64	14	57.1 ± 3.9	397 ± 20

[a] Uteri removed 72 hours after beginning of injection.

TABLE III.—ANALYSIS OF ECP RESPONSE CURVES OF TABLE II

	Uterine Weight	Uterine Ratio
One Dose		
Standard Deviation (s)	10.85	76.22
Slope (b)	37.87	275.42
Index of precision (λ)	0.2866	0.2734
Three Doses		
Standard Deviation (s)	12.49	80.22
Slope (b)	44.98	305.33
Index of precision (λ)	0.2779	0.2620

standard deviations (s), and indexes of precision (λ) shown in Table III. Tests for curvature indicated a linear response over the dose range used. The results in Table III show a similarity in response for single and multiple dose schedules. The standard deviations, slopes, and indexes of precision using either method compare favorably.

Estrone U.S.P. and estradiol U.S.P. were used as examples to compare the effects of ECP to that of more rapidly acting estrogens. Table IV shows that single doses of either estrone or estradiol, with uterine removal 72 hours later, have little effect, whereas multiple doses given over a 3-day period produce significantly larger uteri.

TABLE IV.—COMPARISON IN UTERINE RESPONSE TO ESTRONE U.S.P. AND ESTRADIOL U.S.P. GIVEN IN A SINGLE OR IN THREE SUBCUTANEOUS INJECTIONS[a]

Total Dose, mcg.	No. Mice	Mean Uterine Weight, mg.
Estrone, One Dose		
0.00	9	10.7
0.08	5	10.8
0.16	5	10.8
0.32	22	14.2
0.64	22	14.8
Estrone, Three Doses		
0.00	8	11.7
0.0938	6	26.7
0.1875	6	50.1
0.375	6	61.3
0.75	6	69.7
Estradiol, One Dose		
0.00	5	7.7
0.01	5	7.9
0.03	5	9.4
0.09	5	11.4
0.27	5	11.1
Estradiol, Three Doses		
0.01	5	14.3
0.03	5	26.1
0.09	5	35.2
0.27	5	37.0

[a] Uteri removed 72 hours after beginning of injection.

The prolonged effect of ECP on the mouse uterine weight permits determination of potency by a single injection method. To test the validity and reproducibility of this method, replicate assays using both the uterine weight and uterine ratio methods were done on a preparation containing ECP assayed against a house standard of ECP.

The data of Table V show that results of replicate assays are comparable when either uterine weights or ratios are used. The averages for potency, index of precision, and standard error were similar. Assay No. 4 exceeded the tabular value of 4.03 for the test of parallelism (F) at $P = 0.05$ using uterine weights. However, the same assay using ratios had a relatively high, although acceptable, value of 3.59.

Tests for homogeneity of log potencies of indi-

TABLE V.—COMPARISON IN REPLICATE ASSAYS OF A SINGLE ECP SAMPLE USING UTERINE WEIGHTS, UTERINE RATIOS, AND COVARIANCE

Assay No.	Dosage levels mcg.	Potency, % of House Standard	Slope, b	Standard Deviation, s	Index of Precision, λ	Standard Error, %	Test for Parallelism, F
			Uterine Weight Method				
1	0.16–0.32	86	47.92	11.24	0.2346	15.6	0.87
2	0.16–0.32	90	23.92	6.99	0.2922	19.4	0.96
3	0.12–0.24	126	44.20	10.20	0.2308	15.3	2.42
4	0.12–0.24	114	50.51	9.91	0.1962	13.0	4.24[a]
5	0.12–0.24	97	46.35	5.42	0.1169	7.7	0.00
Averages		103	42.58	8.75	0.2141	14.2	...
			Uterine Ratio Method				
1	0.16–0.32	85	365.41	83.40	0.2282	15.2	2.91
2	0.16–0.32	90	271.70	67.60	0.2488	16.5	0.93
3	0.12–0.24	113	349.36	75.78	0.2169	14.4	3.32
4	0.12–0.24	114	336.48	59.64	0.1772	11.8	3.59
5	0.12–0.24	104	287.07	32.95	0.1148	7.7	0.05
Averages		101	322.0	63.87	0.1972	13.1	...
			Covariance				
1	0.16–0.32	82	45.68	10.72	0.2347	15.6	3.26
2	0.16–0.32	90	24.55	6.40	0.2607	17.3	1.34
3	0.12–0.24	102	42.77	9.15	0.2165	14.4	4.62[a]
4	0.12–0.24	115	54.66	9.56	0.1749	11.6	3.54
5	0.12–0.24	100	46.86	5.41	0.1154	7.7	0.17
Averages		98	42.80	8.25	0.2004	13.3	...

[a] Fail "F" test at $P_{0.05}$.

TABLE VI.—MOUSE UTERINE WEIGHT ASSAYS ON PREPARATIONS CONTAINING ECP

Preparation No.	% of Label Potency	Log Confidence Interval, L	Approx. S.E., %	Slope, b	Standard Deviation, s	Index of Precision, λ
1	118	0.1372	8	55.33	4.88	0.0882
2	101	0.3130	18	35.71	7.21	0.2019
3	97	0.3130	18	35.71	7.21	0.2019
4	92	0.2236	13	47.78	7.14	0.1494
5	91	0.2236	13	47.78	7.14	0.1494
6	98	0.2050	12	38.55	5.33	0.1382
7	94	0.2050	12	38.55	5.33	0.1382
8	95	0.3020	17	49.31	6.33	0.1284
9	91	0.2060	12	49.87	6.90	0.1451
10	99	0.2050	12	49.87	6.90	0.1451
Averages	98	0.2333	14	44.85	6.43	0.1486

vidual assays by both uterine weight and ratio methods were performed; no evidence of heterogeneity was found at $P_{0.05}$ level of significance. Weighted mean potencies, calculated by weighting each log potency inversely as its variance, gave mean potencies of 103 and 101% for uterine weights and ratios, respectively. Since the use of uterine weights and ratios gave very similar results, an analysis of covariance was used to determine whether this would give appreciably better assay precision. Table V shows results using uterine weight as the variate (y) and body weight as the covariate (x) in a covariance analysis. The assumption that body weight had an important influence on uterine weight was not borne out. Although the mean standard error for the covariance method was comparable to that by the ratio method and slightly smaller than that by the weight method, this small increase in precision was not felt to warrant obtaining body weights for subsequent assays.

Numerous preparations containing ECP were assayed by the single dose-mouse uterine weight method. The assays were calculated using statistical methods as outlined in U.S.P. XVI (3) for 2 × 2 balanced assay designs. Table VI shows the results of representative assays of 10 preparations containing ECP assayed at dosage levels of 0.12 and 0.24 mcg., with eight mice at each dosage level. When more than one sample was assayed against a common standard, calculations for the joint assay of several preparations were used (4).

An average log confidence interval (L) of 0.2333 was obtained for the 10 preparations shown in Table VI. Using the value of 0.1486 for the average index of precision (λ), and eight mice per dose, the average standard error of potency was calculated as 12%.

No heterogeneity of assay variance (s^2) was found at $P_{0.05}$ for the independent assays of Table VI when analyzed using Bartlett's test (5). There was also no evidence of heterogeneity of assay slopes for the same group of assays, as determined using the chi square test at the same level of significance.

SUMMARY AND CONCLUSIONS

The method of Rubin, *et al.* (1), using the immature mouse for the bioassay of estrogens has been modified for the assay of a long acting estrogen, estradiol 17-cyclopentylpropionate (ECP). The modifications consist of administering the hormone in a single subcutaneous dose, removing the uteri 72 hours later, and using uterine weights as responses. The results were analyzed according to U.S.P. XVI (3) for a 2×2 balanced assay. When eight mice were used on each dose, the average log confidence interval was 0.2333.

Similar results were obtained by injecting ECP in single or divided doses. This was not the case with estrone or estradiol which were much more effective when multiple injections were given.

It is suggested that the method described for the assay of ECP may be utilized as a rapid bioassay for other long acting estrogens. It may also be used for identification of long acting estrogens and to differentiate between those that are either long or short acting.

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(4) Bliss, C. I., *Drug Standards*, **24**, 61(1956).
(5) Snedecor, G. W., "Statistical Methods Applied to Experiments in Agriculture and Biology," 5th ed., Iowa State College Press, Ames, Iowa, 1956, pp. 285–289.

Liquefaction Time of Rectal Suppositories

By IVO SETNIKAR and SERGIO FANTELLI

An apparatus reproducing the conditions of the environment of the rectum is described. With this apparatus, the liquefaction time of rectal suppositories, either with fatty or water-soluble bases, can be measured. The liquefaction times found for a series of suppositories with fatty bases and with so-called water-soluble bases are reported. It is proposed that a maximum limit should be established for the liquefaction time of rectal suppositories carrying drugs with general action.

The United States Pharmacopeia XVI defines suppositories as "solid bodies of various weights and shapes, adapted for introduction into orifices of the human body and usually melting, softening, or dissolving at body temperature."

However, no method is described for testing these characteristics on the whole suppository. The method for the determination of the melting temperature of fatty (class II) substances is useful for suppository bases but not always applicable to the nonhomogeneous drug-base mixtures of which suppositories are often made. Since the drugs present may alter the melting point (m. p.) of the bases and since the m. p. of the drug-base mixtures may change considerably over a period of time (1), the determination of the m. p. of the suppository base alone is clearly not sufficient for the control of the characteristics mentioned in the U.S.P. definition of suppositories.

Several researchers have felt the need and proposed methods by which these characteristics, and particularly the m. p. can be controlled on the whole suppository. These methods can be classified into two groups: (*a*) m.p. determinations in a dry environment (2, 3) and (*b*) m.p. determinations on suppositories in a water bath (4–7).

With both kinds of methods the m.p. is measured in an environment very different from that of the rectum, for the latter is neither anhydrous nor aqueous. Both give useful information on fatty bases only, because almost all of the so-called water-soluble bases melt at a temperature above body temperature with the first technique, while they dissolve independently of the temperature with the second. Further, with neither method is it possible to record a very important datum, i. e., a representative time of melting, softening, or dissolving at body temperature.

The knowledge of this time is essential in suppositories which include a drug for a general action and must, therefore, be absorbed. Indeed, a suppository which takes too long to liquefy may be expelled before liquefaction, together with the drug it includes. Beside this, it may exert a mechanical irritant action on the rectal ampulla even if the base and the drug, *per se*, are not irritant. The U. S. P. should,

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therefore, recommend, for suppositories including a general-acting drug, a maximum liquefaction time, just as it fixes maximum times for the disintegration of tablets. Also, as in the case of the latter (U. S. P. XVI, p. 830), it should emphasize that "sure disintegration (or rather sure liquefaction) is an essential attribute for suppositories including a general acting drug."

Obviously, if an apparatus is to give a reliable indication on the liquefaction of rectal suppositories, it must reproduce the mechanical and physicochemical conditions of the rectum as faithfully as possible.

The human rectum, or distal portion of the intestine, is 12–14 cm. long and has a maximum diameter of 5–6 cm. The following conditions are present: (*a*) In healthy subjects the average temperature (8) is 36.9° (from 36.2 to 37.6°). (*b*) Water is not present in the liquid state; the feces are semisolid and contain 77–82% water (9). (*c*) Water may issue from the blood through a process of osmosis. Although the rectal mucosa tends to absorb water and transfer it to the blood, it may be regarded as a semipermeable membrane since it also permits the passage of water in the opposite direction, provided that there are osmotic forces to attract it. This process is, however, antiphysiological and may be both irritating and painful. (*d*) There are practically no peristaltic movements (10) to churn and mix up the contents of the ampulla of the rectum. (*e*) The pressure on the contents of the rectum is, according to posture, from 0 to 50 cm. water (10, 11). (*f*) Feces may be present.

Nearly all these conditions (temperature, heat transmission, semipermeability of the walls, pressure) may be reproduced in a semipermeable cellophane dialyzer tube immersed in a water bath at 37.0°.

EXPERIMENTAL

Apparatus.—The apparatus (Fig. 1) consists of a glass cylinder with an external diameter of 50 mm., narrowing down to 22 mm. at either end for a length of 30 mm. The cylinder is fitted with two connections through which water circulates from a circulating water pump, thermoregulated to 37.0°.

The apparatus is prepared in the following way: a 34–35-cm. length of cellulose dialyzer tubing, size inflated diameter 1 1/8 in.,[1] is moistened, opened, and placed in the glass cylinder. The tube is drawn out of either end of the cylinder and secured thereto with two elastic bands. Then the rubber tubes of the thermoregulated pump are attached to the connections of the apparatus and water is made to circulate in it. The apparatus is raised or lowered, keeping it vertical, until the level is found at which the lower half of the cellophane tube is collapsed and the upper half gaping. At the level at which the tube starts to close in, the hydrostatic pressure of the water in the apparatus is about zero.

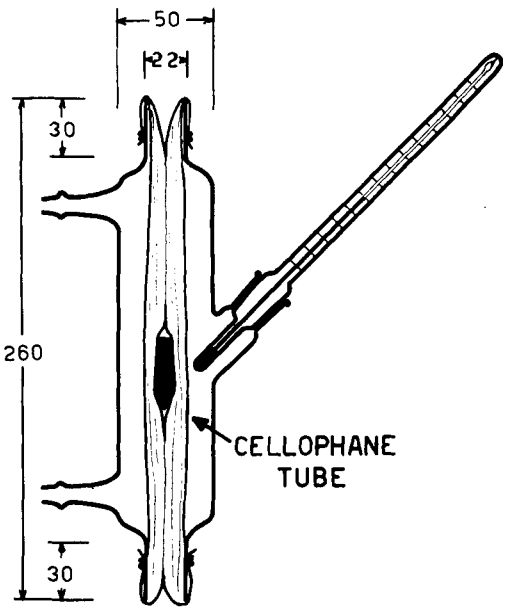


Fig. 1.—Apparatus for measuring the liquefaction time of rectal suppositories; the dimensions are in millimeters. The thermometer scale is divided into tenths of a degree and a scale ranging from 32 to 45° is adequate.

When the thermometer reaches a stable temperature of 37.0°, a suppository is dropped in, the apparatus is lowered about 30 cm., and measurement of the liquefaction time begins.

If the suppository contains a small quantity of drug, or if the drug is dissolved in the base, one can gauge to a high degree of accuracy the moment in which the suppository has entirely liquefied. When the suppository contains large quantities of undissolved drugs, e.g., 0.5 Gm. of aminophylline, it is more difficult to determine the moment of complete liquefaction. In this case it may be helpful to raise the apparatus every 2–3 minutes so as to open up the cellophane tube at suppository level in order to facilitate dispersion of the material already liquefied. Another device is to place a metal spiral, with a diameter of about 5 mm. and a pitch of 3–5 mm., near the suppository in order to promote upward and downward dispersion of the liquefied material. In the most difficult cases, the difference in the assessment of the moment of complete liquefaction by independent observers does not exceed 10–30 seconds.

The apparatus can also be used for the determination of the m.p. of suppositories made by water-insoluble bases. For this purpose the temperature is raised at a speed of 1° every 10 minutes until the suppository melts.

When the experiment is over, the apparatus is raised until the cellophane tube gapes, the inner walls of the tube are washed with water containing a detergent, then with distilled water, and allowed to drip for a few minutes. The apparatus is then ready for use again.

[1] Fisher Catalog No. 8–667.

Results.—Suppositories consisting of commercially available bases, the compositions of which are reported by Pennati and Steiger-Trippi (12), were prepared and kept at room temperature for 1–6 months. The liquefaction time was then measured and the results are given in Table I.

The error of observation was determined by repeating the measurements of ten identical suppositories. For the theobroma oil base the follow-

TABLE I.—MELTING POINT BY THE METHOD OF BOGS AND LIQUEFACTION TIME AT 37.0°

Base	M. P., °C.	Liquefaction Time, min.	Supplier
		Fatty bases	
Theobroma oil	34	6.09	
Caol TR	39	[a]	Sarn, Novara, Italy
Caol TR 38/40	43	[a]	Sarn, Novara, Italy
C.B.S.A. "N"	37	8	Calvè, Delft, Holland
Dehydag III	39	[a]	Deutsche Hydrier-Werke
Dehydag IV	38	[a]	Deutsche Hydrier-Werke
Estarinum A	37	8.58	Edelfettwerke, Hamburg, Germany
Estarinum B	38	30	Edelfettwerke, Hamburg, Germany
Estarinum B B	36	6	Edelfettwerke, Hamburg, Germany
Estarinum C	40	[a]	Edelfettwerke, Hamburg, Germany
Estarinum D	45	[a]	Edelfettwerke, Hamburg, Germany
Estarinum P1	37	10.66	Edelfettwerke, Hamburg, Germany
Imhausen E	38.5	[a]	Imhausen Co., Witten
Imhausen ET	39	[a]	Imhausen Co., Witten
Imhausen H	36	9	Imhausen Co., Witten
Imhausen OG	36.5	7.42	Imhausen Co., Witten
Imhausen W	36.5	10.25	Imhausen Co., Witten
Imhausen WN	39	[a]	Imhausen Co., Witten
Lipomassa C.F.M.	39	33	C.F.M., Milano, Italy
Neo Suppostal	41	75	Medifarma, Milano, Italy
Suppostal ES	45	[a]	Medifarma, Milano, Italy
Suppocire A	37	10.5	Gattefosse, Lyon
Suppocire B	38	[a]	Gattefosse, Lyon
Suppocire C	39	[a]	Gattefosse, Lyon
Suppolanol	35	5.33	Esperis, Milano, Italy
Vi-Tin 136	37	8	Treves, Torino, Italy
Vi-Tin 138	39	[a]	Treves, Torino, Italy
		Water-soluble bases	
Carbowax 1540	46	18	Union Carbide, New York
Carbowax 4000	59	30	Union Carbide, New York
Carbowax 6000	62	45	Union Carbide, New York
Carbowax 6000 + 20% H_2O	54	27	Union Carbide, New York
Glycerinated gelatin, U.S.P.	50	32	
Idromassa C.F.M.	60	40	C.F.M., Milano, Italy
Idropostal	60	37	Medifarma, Milano, Italy
Idropostal 90% + liquid Idropostal 10%	58	35	Medifarma, Milano, Italy
Idropostal G 60% + Glycerol 40%	52	33	Medifarma, Milano, Italy
Idropostal M 90% + H_2O 10%	60	35	Medifarma, Milano, Italy
Idrorectonal H	54	[a]	Frat. Giacomini, Milano, Italy
Idrorectonal HL	41	[a]	Frat. Giacomini, Milano, Italy
Idrorectonal W	55	[a]	Frat. Giacomini, Milano, Italy
Massa Neutralis C.F.M.	39	40	C.F.M., Milano, Italy
Myrj 52	51	31	Atlas Powder Co.
Neutril	59	39	I.C.V., Como, Italy
Tween 61	39	[a]	Atlas Powder Co.

[a] Softening after 10–20 minutes, no liquefaction during 1 hour.

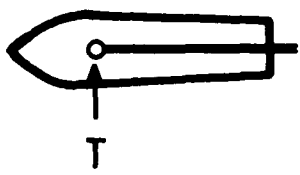


Fig. 2.—Actual shape and size of the suppositories used. *T* indicates the position of the thermocouple inserted in the suppository to measure the speed of heating.

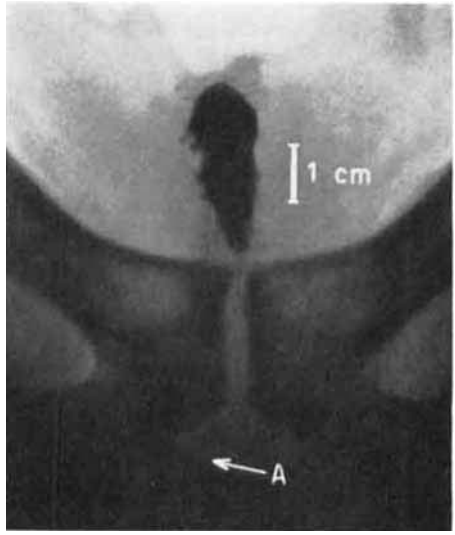


Fig. 3.—Roentgenograph of the pelvic inlet of 34-year-old man, in the supine position, 5 minutes after insertion of a type A suppository. The suppository has completely disintegrated. Arrow *A* marks the position of the anal orifice, to which in this and in the following figures, a lead strip piece was juxtaposed. The dimension of 1 cm. on the plane of the suppository is also marked. The size and shape of the suppository were the same as in Fig. 2.

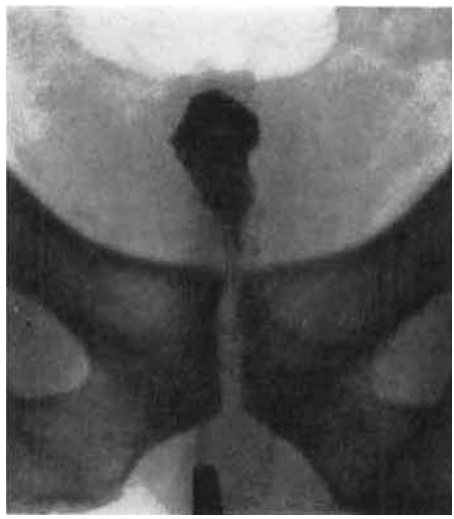


Fig. 4.—Type A suppository, 10 minutes after insertion. The contrast medium tends to spread and rise up the rectum.

ing results were obtained: average liquefaction time 245 seconds, standard deviation ± 10 seconds (4.1% of the average time), standard error 3.2 seconds (1.3% of the average time). Comparable percentage standard deviation and errors were obtained for other bases. The data naturally depend, to some extent, upon the size and shape of the rectal suppositories used. The ones used in these experiments are reproduced in their actual size in Fig. 2.

To check whether the values found by the *in vitro* method were representative of what actually occurs in the ampulla of the human rectum, two types of suppositories were prepared with the following compositions: *Type A Suppositories (Fatty Base).*—theobroma oil, 1.6 Gm.; barium sulfate, 0.5 Gm.

Type B Suppositories (Water-Soluble Base).—Carbowax 1540, 0.8 Gm.; Carbowax 6000, 1.1 Gm.; Polyethylene glycol 400, 0.5 Gm.; barium sulfate, 0.5 Gm.

These suppositories were of the size and shape represented in Fig. 2. The liquefaction time of these suppositories was tested on five healthy male subjects kept throughout the experiment in a supine position. The pelvic inlet was roentgenographed at set intervals. The pictures obtained in one of these experiments are reproduced in Figs. 3–9 and the results summarized in Table II. The agreement between the *in vitro* and *in vivo* results is sufficiently satisfactory to show that the proposed method represents the course of events within the ampulla of the human rectum.

TABLE II.—LIQUEFACTION TIMES *In Vitro* AND *In Vivo*

	Softening Temp.,[a] °C.	M.P.,[a] °C.	Liquefaction Time *in vitro* min.	Liquefaction Time in the Human Rectum, min.
Suppository A	30	34	4.5	between 3 and 7
Suppository B	52	58	40	between 30 and 50

[a] Determined by a modified Bogs method.



Fig. 5.—Suppository type A, 15 minutes after insertion. The upward movement and spread of the contrast medium are even more marked.

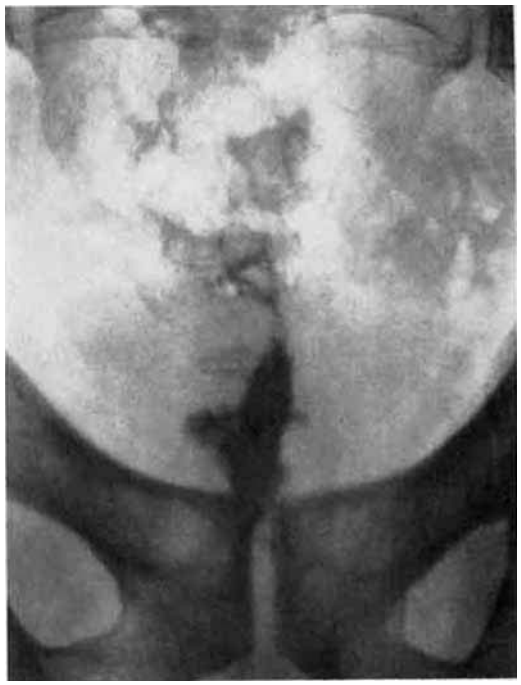


Fig. 6.—Roentgenograph of the pelvic inlet of the same subject as that of Fig. 3, 10 minutes after insertion of a type B suppository. The disintegration of the suppository and the spread of the contrast medium have hardly begun.

When the apparatus is used for the determination of the m.p. of fatty-base suppositories it must be known at what rate the temperature of the suppository comes into equilibrium with that of the apparatus in order to regulate the speed at which the temperature of the apparatus should be raised. For this purpose, suppositories of Imhausen H were prepared and a thermocouple was inserted at the level marked T in Fig. 2 (Imhausen H is a mixture of hydrogenated natural fats, mostly triglycerides of laurinic acid, with a monoglyceric ester as emulsifier). One suppository prepared in this way was put in the apparatus kept at 34.7°. The temperatures measured by the thermocouple in the center of the suppository during 1 hour of experiment are given in Fig. 10*A*.

As the rise in temperature (dT) in the time (dt) is, in all probability, proportional to the difference between the environment temperature (T_e) and the temperature of the suppository (T), the following equation may be written

$$dT/dt = k(T_e - T) \qquad \text{(Eq. 1)}$$

where k is a constant depending on the heat capacity and conductivity of the suppository. By integration, Eq. 1 becomes

$$kt = \log (T_e - T_0)/(T_e - T) \quad \text{(Eq. 2)}$$

where T_0 expresses the temperature of the suppository at the beginning of the experiment.

Equation 2 shows that plotting the logarithm of $(T_e - T_0)/(T_e - T)$ on the ordinate and the time on the abscissa of a diagram of Cartesian axes, a straight line should be obtained with a slope equal

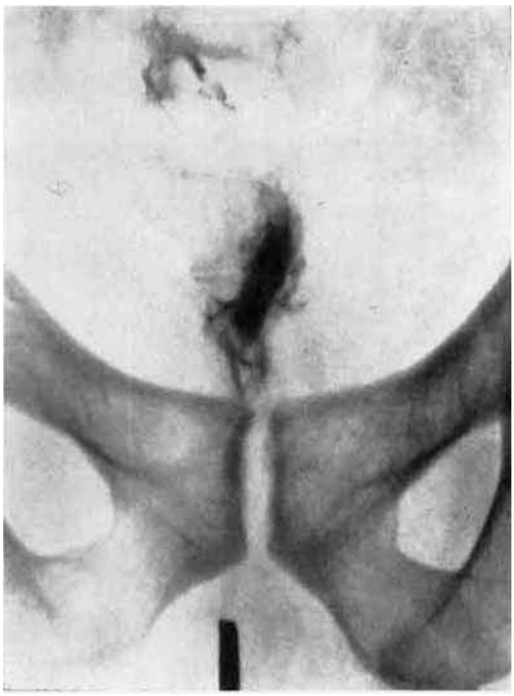


Fig. 7.—Type B suppository, 30 minutes after insertion. The disintegration of the suppository and the spread of the contrast medium are already very evident.

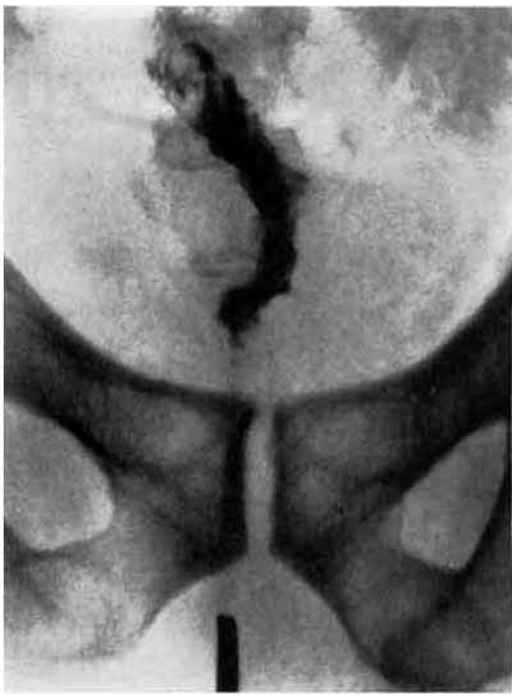


Fig. 8.—Type B suppository, 45 minutes after insertion. Disintegration is complete.

to k. The data of Fig. 10*A* were recalculated in this way (in this experiment T_e was equal to 34.7° and T_0 equal to 18.3°) and the results are reported in Fig. 10*B*.

One notes that the rise in temperature proceeds at first at a higher speed ($k = 0.141$) and then, when in all likelihood a more uniform temperature gradient has formed throughout the thickness of the suppository, the rise proceeds more slowly ($k = 0.053$). The half-equilibrium time is reached in 2 minutes, the 99% equilibrium time in 27.5 minutes.

It may also be calculated that, when the temperature in the apparatus is raised by 1° every 10 minutes, the temperature at the center of the suppository remains behind that of the apparatus by 0.1–0.3°. Under these conditions the error of appreciation of the m.p. is smaller than 0.3°. If one considers that when the suppository begins to melt its diameter is reduced and the transmission of heat is facilitated, the difference between the temperature inside the suppository and that of the environment becomes still smaller and likewise the error.

This method of analysis of the temperature transmission may be useful also in order to know how size, method of preparation, and composition may affect the temperature-equilibrium time and to choose the best conditions from this point of view.

DISCUSSION

In Table I the bases studied are divided into fatty and water-soluble, though the distinction is not always accurate because many commercial bases consist of mixtures of fats and more-or-less water-



Fig. 9.—Type B suppository, 60 minutes after insertion. Disintegration is complete and the spread of the contrast medium considerable. If this figure is compared with Fig. 5, it will be seen that the spread of the contrast medium is greater. This is probably due to the longer time (15 minutes in Fig. 5 and 60 minutes in Fig. 9). In this figure the contrast medium begins at a distance of about 4 cm. from the anal orifice and extends for about 5 cm. up the rectum. In 60 minutes it has risen on the average about 2 cm. up the rectum.

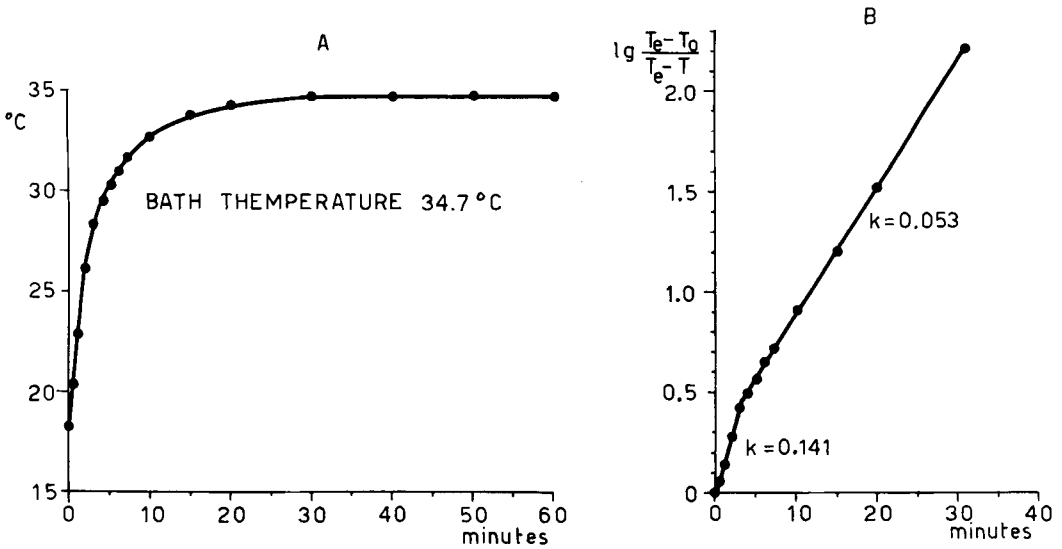


Fig. 10.—Speed of the rise in temperature at the center of the suppository measured with a thermocouple situated in the way described in Fig. 2. The data of Fig. 10*A* are transferred on to Fig. 10*B* to convert Eq. 2 of the text into a linear equation in order to calculate the constant, *k*, of the speed by which the temperature in the center of the suppository increases.

soluble substances which help the fats to emulsify and contribute to the liquefaction process. Indeed, if the base is purely fatty as, e.g., theobroma oil, in order to liquefy it must have a m.p. of 37° or less. This does not happen with Neo Suppostal and Lipomassa C.F.M., which liquefy in the cellophane tube even though they have a m.p. higher than 37°. Therefore, to know whether suppositories liquefy in the rectum, it is not enough to know the m.p. only, even of the so-called fatty bases. Further, as the m.p. of the so-called water-soluble bases is higher than 37°, it must be concluded that, for rectal suppositories, the m.p. tells us very little about the capacity of bases for liquefaction in the rectum.

On the strength of their high m.p., suppositories made with water-soluble bases may, at first sight, seem peculiarly suited to hot or tropical climates. Actually, however, other factors have to be borne in mind, i.e., that liquefaction often takes some considerable time (although it can be shortened by the addition of drugs, propylene glycol, water, etc.) and that these bases dissolve by attracting water from the blood by an antiphysiological process of osmosis which may be irritating and painful. Other disadvantages of these bases have already been noted by Czetsch-Lindenwald (13). Suppositories with water-soluble bases should, therefore, be always subjected to extensive pharmaceutical and biological testing.

We would like also to comment on the definition of suppositories given in the U. S. P. The term "softening" is vague, and the terms "melting" or "dissolving" refer to the base rather than to the suppository, for the drug included does not always melt or dissolve. The reference to "body temperature" is not applicable to dissolving, which depends rather on the presence of water. Further, it seems to us that for rectal suppositories a distinction should be made between those with a local action, e.g., glycerinated gelatin suppositories U. S. P., for which the softening or liquefying is not always essential, and those including a drug for a general action, release of which depends in first instance on the liquefaction of the suppository.

For these suppositories, the liquefaction time is of such great biological importance that a maximum limit should be fixed for it, e.g., 10 minutes, as proposed by Czetsch-Lindenwald (14).

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Characterization and Mode of Degradation of the Neurophysiologically Important Transaminase Inhibitors, the α-Aminooxyalkanoic Acids

By EDWARD R. GARRETT†

The α-aminooxyalkanoic acids such as aminooxyacetic acid have shown extraordinary properties of inhibition of the γ-aminobutyric acid-α-ketoglutaric acid enzymatic transamination to glutamate-succinic semialdehyde *in vivo* whereas the β-aminooxyalkanoic acids such as β-aminooxypropionic acid are of low potency. The unexpected lack of significant basicity of the aminooxy group in aminooxyacetic acid and its nondipolar ion nature implies a cyclic form in solution which may be responsible for its ability to cross cell walls and exhibit *in vivo* activity. The aminooxyacetic and β-aminooxypropionic acids are degraded in mild alkali at 60° to form ammonia and nitrite ion in the ratio of 2:1, and a mechanism of hydroxylamine release is postulated to account for this stoichiometry where the rate determining step is the release of hydroxylamine and a carboxylic acid. Hydroxylamine itself is quickly transformed by mild alkali at 60° to ammonia and nitrite ion. This mode of degradation is significant since hydroxylamine itself is a known transaminase inhibitor.

THE IMPORTANCE of the enzymatic transamination of γ-aminobutyric acid, GABA, with α-ketoglutaric acid to form glutamate and succinic semialdehyde in brain tissue and the potential effect on neurological activity has been stressed in the literature (1–3). Thus, inhibition of this transamination is of interest in the possible correlation of GABA levels in the brain with physiological properties such as definite depression of the central nervous system (4). Although suitable doses of hydroxylamine have been shown to increase the GABA levels in the brain of rats (5), a potent inhibitor *in vitro* and *in vivo* of the transaminase involved has been recently shown by Wallach (4) to be aminooxyacetic acid (AOAA), H_2NOCH_2COOH. Recent studies on various analogs have shown that good *in vivo* activity was generally limited to the α-aminooxyalkanoic acids or their easily hydrolyzed derivatives such as esters (6). None of the compounds studied was superior to aminooxyacetic acid (6).

The studies reported in this paper include the titrimetric characterization, stability, and possible modes of degradation of two representative compounds, aminooxyacetic acid and β-aminooxypropionic acid. The purposes were to obtain data for the possible rationalization of the observed difference in *in vivo* activity of these compounds.

Received August 7, 1961, from the Research Division, The Upjohn Co., Kalamazoo, Mich.

Accepted for publication August 23, 1961.

The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance. Grateful appreciation is given to Dr. R. V. Heinzelman for discussions which led to many of the rationales expressed in this paper.

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EXPERIMENTAL

Purity by Titration.—Aminooxyacetic acid hemihydrochloride (7) ($H_2NOCH_2COOH.{}^1/_2HCl$, mol. wt. = 109.3) 0.10 mmole, was dissolved in 10.00 ml. solvent and was titrated with 2.000 *N* sodium hydroxide in the Cannon automatic titrator (8) (see Fig. 1). A minor inflection (pH 3.4) was observed at an amount of titer which was $^1/_3$ of the total titer consumed up to a large inflection (pH 6–10). This was equivalent to the $^1/_2$ HCl per molecule. The titer between the minor and major inflections was equivalent to the number of moles.

Fifteen titrations showed that the material was of high purity by titration, 100.0 ± 0.3%.

Similarly, 10.00 ml. of 0.0050 *M* β-aminooxypropionic acid hydrochloride (9) ($H_2NO—CH_2CH_2COOH.HCl$, mol. wt. = 141.6) was titrated (see Fig. 2). The minor inflection was at pH 4.2, and 8 titrations conducted in water and in various aqueous alcohol solvents showed that the material was of high purity by titration, 100.0 ± 1.0%.

The apparent pKa values of these compounds as functions of per cent ethanol by volume are given in Table I. These pKa values are estimated from the pH at half-neutralization of the apparent prosthetic groups.

The compounds studied were provided by E. L. Schumann (6) of these laboratories.

Stability at 60° in Acid and Neutral Regions.—Solutions of 0.01 *M* aminoooxyacetic acid hemihydrochloride as studied up to 554 hours in water (pH 2.75), up to 22 hours at pH 9.5, up to 280 hours in 0.1 *N* hydrochloric acid at pH 1.20 were completely stable as evidenced by no change in the titration curves. Solutions of 0.005 *M* β-aminooxypropionic acid hydrochloride as studied up to 300 hours at pH 8.0 and up to 280 hours in 0.1 *N* hydrochloric acid at pH 1.20 were also completely stable by titration. No differences were observed in the shape of the curves or the apparent equivalent weight for aliquots taken up to these stated times.

Although β-aminooxypropionic acid hydrochloride in aqueous solution *ca.* pH 5.1 was stable up to the

TABLE I.—pKa AS FUNCTIONS OF PER CENT ETHANOL BY VOLUME

	Aminooxyacetic Acid			β-Aminooxypropionic Acid		
% Ethanol =	0	50	95	0	50	95
pKa_1	2.75	2.95	2.91	3.65	3.93	4.15
pKa_2	4.50	4.88	6.30	5.22	5.68	7.40

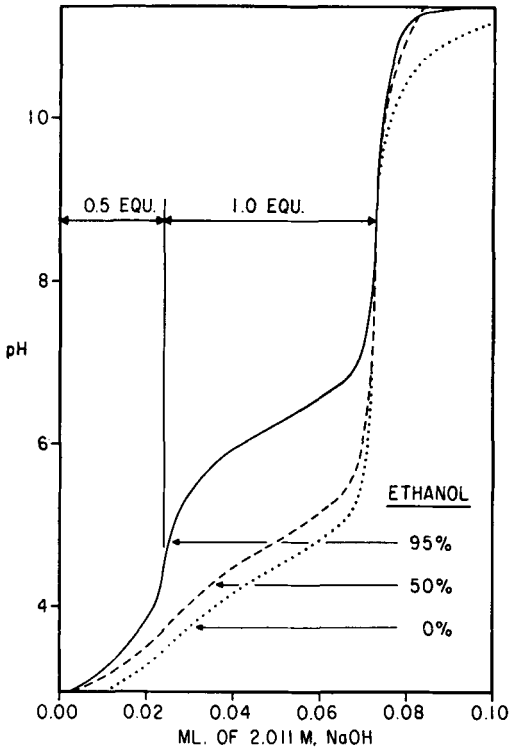


Fig. 1.—Potentiometric titrations of 0.010 *M* aminooxyacetic acid hemihydrochloride in varying per cent ethanol-water solutions.

17 hours studied at room temperature by titration, a change in the titration curve was observed when the solutions were subjected to 60°. This had not been observed with aminooxyacetic acid hemihydrochloride in aqueous solution at 60°. The change in the titration curves resulted in an apparent increase in pKa_2 from 5.22 to 6.0 at 100 hours with a significant decrease in the amount of titer necessary to reach the major inflection at pH 8 by 5% at 18 hours and 10% by 100 hours. This same phenomenon occurred when β-aminooxypropionic acid was subjected to pH 4 at 60°.

Stability in Alkaline Media.—Degradation of the aminooxyalkanoic acids occurred in mild alkali at 60°. Ten-milliliter aliquots of 0.0101 *M* sodium aminooxyacetate in 0.01 *M* sodium hydroxide were acidified with 1 ml. 0.25 *N* hydrochloric acid and potentiometrically titrated with 2.00 *M* sodium hydroxide. In another study, aliquots were directly titrated with 1.904 *M* hydrochloric acid. Typical curves of such titrations as a function of time of subjection to 60° are given in Fig. 3 and pertinent information is tabulated in Table II.

Similarly, typical curves for the titrations with time by 1.904 *M* hydrochloric acid of 10-ml. aliquots of 0.01 *M* sodium β-aminooxypropionate maintained at 60° in 0.01 *M* sodium hydroxide are given in Fig. 4 and pertinent information for such a degradation is given in Table III.

TABLE II.—TITRATION OF 10-ML. ALIQUOTS OF 0.0101 *M* SODIUM AMINOOXYACETATE IN 0.010 *M* SODIUM HYDROXIDE MAINTAINED AT 60°

Time of Sample, Hr.	pKa_2 (Estd.)	Estd. pKa of New Function[a]	$10^3 \times$ Molarity of New Function[b]	$10^3 \times$ Molarity of NaOH Consumed
Titration with 2.011 *M* NaOH after Acidification with 1 ml. 0.25 *M* HCl				
0.0	4.47	..	0.0	
16.8	4.45	9.3	0.6	
23.8	4.56	9.3	1.2	
40.8	4.57	9.3	1.5	
141	4.52	9.5	2.8	
283	4.60	9.5	4.0	
Titration Directly with 1.904 *M* HCl				
0.0	4.48	...	0.00	0.0
5.0	4.52	...	0.13	0.0
22.0	4.45	9.40	1.03	0.4
47.7	4.50	9.55	1.90	0.8
74.8	4.55	9.62	2.25	0.8
142	4.60	9.65	3.43	1.3
336	4.55	9.58	4.80	2.3

[a] Estimated from point of half-neutralization of between apparent inflection at pH 10.7 and definite inflection at pH 7 (see Fig. 2). [b] Portions of titration curves above pH 10 are superimposed over zero-time curve. The titer difference between the major inflections at pH 7 permits quantification of the amount of pKa 9.4 material appearing.

Hydroxylamine was also subjected to 0.01 *M* sodium hydroxide at 60° and aliquots were titrated at intervals. The pKa = 6.00 of hydroxylamine disappeared within 10–15 minutes and an apparent pKa of 9.3 appeared with a significant loss of free alkali.

The degraded alkaline solutions of both aminooxyalkanoic acids and hydroxylamine showed the presence of large amounts of nitrite ion as tested by the method of Forist (10).

RESULTS AND DISCUSSION

Ionic Nature of Aminooxyalkanoic Acids.—In general, pKa values of carboxylic acids increase with decreasing aqueous content of miscible solvents, whereas the pKa values of ammonium ions show little change or tend to decrease (11). The rationale is that a decrease in dielectric constant should not favor the dissociation of an uncharged species such as a carboxylic group whereas it should show no effect or slightly favor the dissociation of a protonated amine. This latter dissociative equilibrium does not involve a change in the number of ions. The pKa data of Table I indicate very little change in pKa_1 values with increasing alcohol content, whereas the pKa_2 values increase, and the increase is especially large between 50 and 95% (by volume)

TABLE III.—TITRATION OF 10-ML. ALIQUOTS OF 0.010 *M* SODIUM β-AMINOOXYPROPIONATE IN 0.010 *M* SODIUM HYDROXIDE MAINTAINED AT 60°

Time of Sample, hr.	pKa_1	pKa_2	$10^3 \times$ Molarity of New Function	Estd. pKa of New Function	$10^3 \times$ Molarity of NaOH Consumed
0.0	3.70	5.20	0.00	...	0.0
17.8	3.70	5.25	1.33	9.5	0.36
41.9	3.78	5.20	1.90	9.45	0.95
118	3.82	5.25	3.52	9.63	1.15
180	4.10	5.50	4.38	9.76	2.48

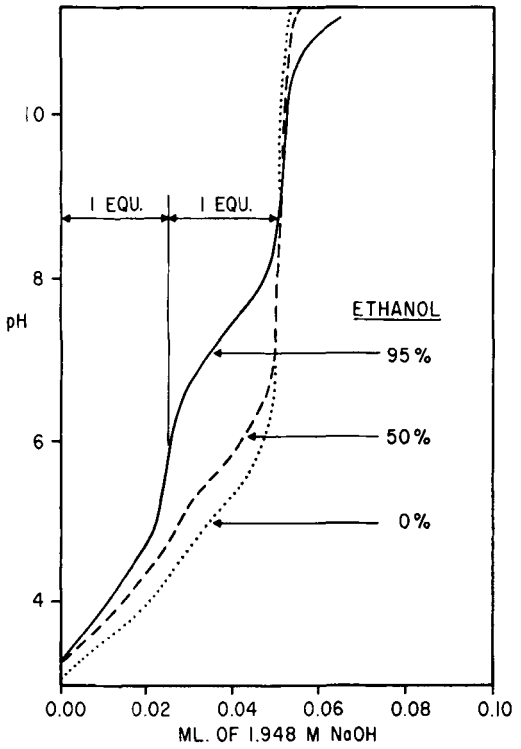


Fig. 2.—Potentiometric titrations of 0.005 *M* β-aminooxypropionic acid hydrochloride in varying per cent ethanol-water solutions.

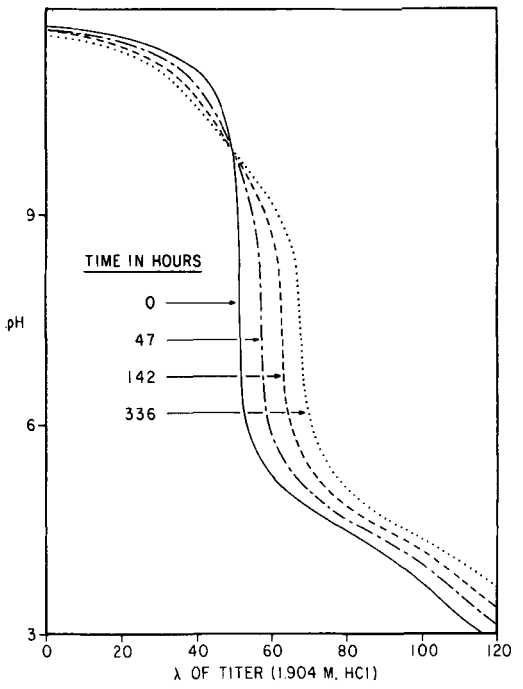
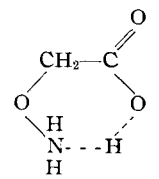


Fig. 3.—Titrations of 0.01 *M* sodium aminooxyacetate in 0.01 *M* sodium hydroxide maintained at 60°. The titer is 1.904 *M* hydrochloric acid.

ethanol content of the solvent. These facts permit the postulation of a nondipolar ion for the aminooxyalkanoic acids in solution, i.e., pKa_1 is assigned to the aminooxy group and pKa_2 is assigned to the carboxyl group. The smaller increase in pKa_2 between 0 and 50% ethanol content of the solvent than between 50 and 95% suggests that unusual molecular interactions may occur in solutions of high dielectric. The presence of a hemihydrochloride is in itself unique and implies that aminooxyacetic acid exists as the dimer in the crystalline form: $Cl^- \cdot NH_3^+—OCH_2COO^- \cdot NH_3^+—OCH_2COOH$.

The apparent pKa_1 values are determined on the premise that pKa_1 = pH at half-neutralization. Actually, the acidity of aminooxyacetic acid hemihydrochloride solutions is no less than what an equivalent molarity of hydrochloric acid would produce and thus strongly indicates that if any aqueous pKa_1 of this compound exists, it is very much less than the 2.75 estimated from half-neutralization and listed in Table I.

These facts indicate that aminooxyacetic acid may exist in cyclic form in solution where the aminooxy group is unavailable for ready protonation, as



If the number of methylene groups is increased between the carbonyl and the aminooxy group, cyclicization is inhibited and the aminooxy group becomes more readily protonated, as in the case of the real pKa_1 of β-aminooxypropionic acid hydrochloride.

Mode of Alkaline Degradation.—When aminooxyacetic acid is subjected to mild alkali and titrations of aliquots are made as a function of time (Fig. 3 and Table II), a new titratable group appears at *ca.* pKa 9.4 and the acidity of the reacting solution increases, indicative of free alkali consumption. The increase in molarity of the new 9.4 pKa func-

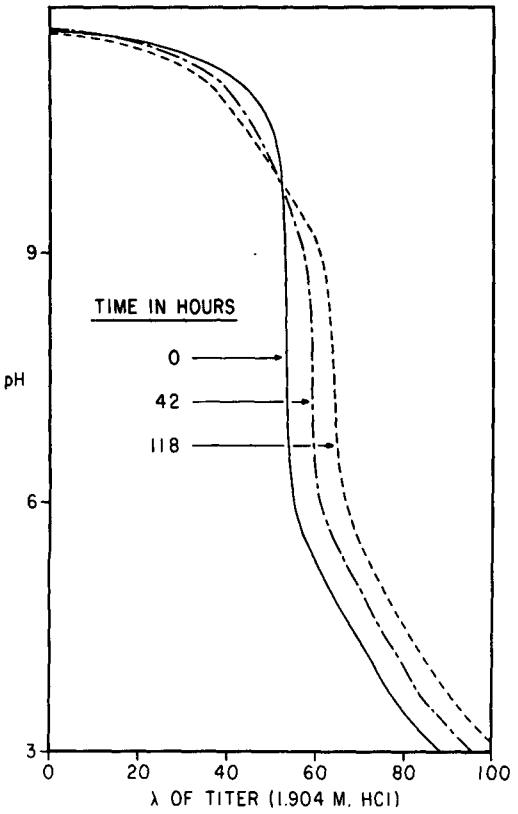


Fig. 4.—Titrations of 0.003 *M* sodium β-aminooxypropionate in 0.01 *M* sodium hydroxide maintained at 60°. The titer is 1.904 *M* hydrochloric acid.

tion was estimated by superimposition of the titration curve above pH 10 and estimating the differences in titer between the major inflection of a titration curve at any time with inflection of the titration curve taken at zero time (12). The loss of free alkali was estimated from differences in standard acid titer necessary to achieve the initial pH (12) and is given in Table II from the data on standard acid titration of aliquots. Standard alkaline titration of acidified aliquots gave variable results and indicated that the strong acid anion produced by basic treatment was unstable in acidic solutions.

These facts were consistent with the assignment of ammonia to the observed pKa 9.4 (NH_3, pKa = 9.25) and nitrite ion (pKa 3.6), produced in the ratio of 2:1 (see Tables II and III). The possible explanation of this degradation in this ratio is given in the following equations

$$H_2NO{-}CH_2COOH \xrightarrow[HOH]{OH^-} H_2NOH + HOCH_2COOH \quad \text{(Eq. 1)}$$

$$3NH_2OH + OH^- \rightarrow NO_2^- + 2NH_3 + 2H_2O \quad \text{(Eq. 2)}$$

Equation 2 predicts the formation of NH_3 and NO_2^- in the ratio of 2:1. The formation of the latter would lower the apparent acidity and consume free alkali. Chemical tests have unambiguously shown the presence of nitrite. Hydroxylamine (pKa = 6.00) was not observed by titration. This indicates that the possible reaction shown in Eq. 2 is the fast one and that reaction shown in Eq. 1 is rate determining. This fact was substantiated by subjecting hydroxylamine to 0.01 *M* sodium hydroxide at 60°. Within several minutes the pKa 6.0 disappeared, a pKa of 9.3 appeared with an alkali consumption in

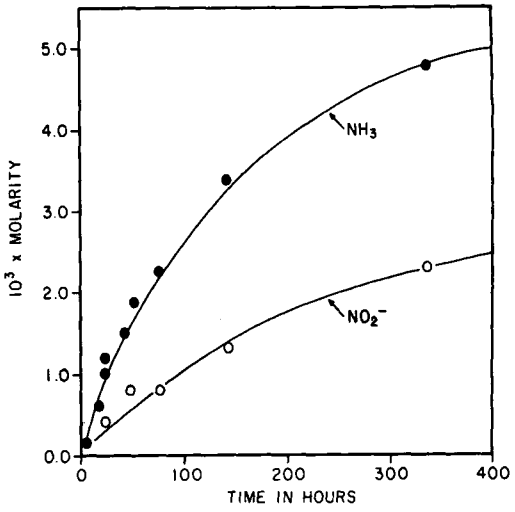


Fig. 5.—Appearance of NH_3 and NO_2^- as a function of time from the degradation of 0.010 *M* sodium aminooxyacetate in 0.010 *M* sodium hydroxide.

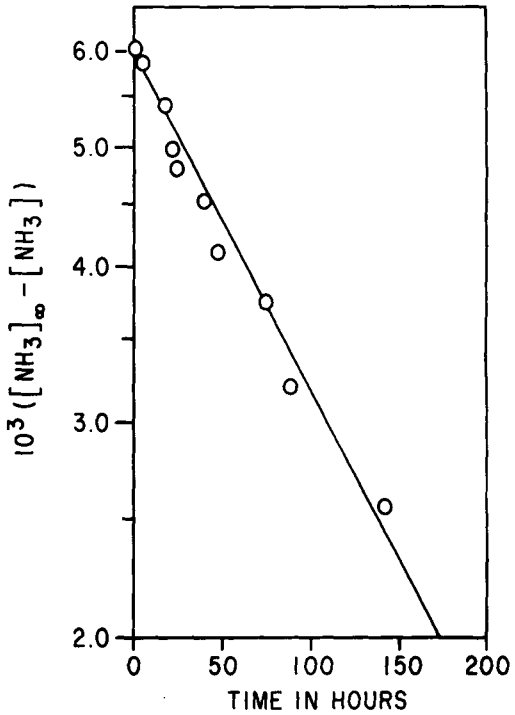


Fig. 6.—First-order plot for the appearance of $[NH_3]$ from the degradation of 0.010 *M* sodium aminooxyacetate in 0.010 *M* sodium hydroxide. The $[NH_3]_\infty$ is taken as 6.0×10^{-3}.

the ratio 1:2 with the pKa 9.3, and nitrite ion was conclusively identified as present.

The same phenomena were observed for the alkaline degradation of β-aminooxypropionic acid (Table III).

It can be concluded that carbonate or hydroxylamine is not an end product of the mild alkaline degradation of aminooxyalkanoic acid (no pKa's 6.0–6.5 are observable) and that the most probable sequence is the hydrolysis to hydroxylamine and some carboxylic acid, with a subsequent fast degradation of hydroxylamine to ammonia and nitrite ion.

Figure 5 plots the ammonia and nitrite produced as a function of time in 0.01 *M* sodium hydroxide at 60° as based on the above discussion. Apparent first-order plots for the production of ammonia and nitrite are given in Fig. 6 for aminooxyacetic acid with an apparent half-life of 110 hours under these conditions.

It is interesting to note that although β-aminooxypropionic acid is quite stable at 60° below pH 2 and between pH 7–10, degradation other than alkaline also occurs with an apparent maximum at pH values of *ca.* 3–4. This implies a significant acid catalyzed hydrolysis of the neutral molecule where

$$H_2NO{-}CH_2CH_2COOH + H_3O^+ \rightarrow H_3\overset{+}{N}OH + HOCH_2CH_2COOH \quad \text{(Eq. 3)}$$

the hydroxylamine was identified by the appearance of a pKa 6.0 since hydroxylamine does not readily degrade at this pH. It may be inferred that at a pH less than 2 the protonated compound, $^+H_3NO{-}CH_2CH_2COOH$, is the major species and resists hydrogen ion attack. At a pH greater than 7, the hydrogen ion and hydroxyl ion concentrations are insufficient for catalytic action.

Biological Activity and the Chemistry of Aminooxyalkanoic Acids.—The increased biological activity of aminooxyacetic acid over all other compounds studied (4, 7) can be assigned to its unusual chemical characteristics which include the absence of any apparent basicity of the aminooxy group in aqueous solution, perhaps due to a tendency to exist in the form of a five-membered ring. This in turn may be responsible for its ability to cross cell walls.

The ability of these α-aminooxyalkanoic acids to release hydroxylamine *in vitro* is of interest in light of the known action of hydroxylamine as an inhibitor of transaminase. This suggests that these α-aminooxyalkanoic acids may uniquely carry and release hydroxylamine at the physiological site, wheras hydroxylamine itself is too readily metabolized or not readily transportable. Of course, the possibility that the intact acids are the primary agent is not refuted.

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Sulfide Derivatives of Cysteine II

Some Sulfonamide Derivatives of Cysteine and Methionine

By MATTHEW VERDERAME

Seven new sulfide derivatives of cysteine have been prepared. In addition sulfonamides of cysteine, L-3-mercapto-N-sulfanilylalanine, and methionine, DL-N-sulfanilylmethionine, among others, have also been prepared. Except for L-3-[(3′-hydroxy-4′-carboxyphenyl)-carbamylmethylthio]-alanine (compound 2), which was slightly effective against a *Klebsiella pneumoniae* infection in Swiss mice, all other compounds tested for various activities showed a lack of physiological effectiveness.

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DISCUSSION

The sulfide derivatives of cysteine, L-3-(substituted)-methylthioalanines (Table I), were made by

the ratio 1:2 with the pKa 9.3, and nitrite ion was conclusively identified as present.

The same phenomena were observed for the alkaline degradation of β-aminooxypropionic acid (Table III).

It can be concluded that carbonate or hydroxylamine is not an end product of the mild alkaline degradation of aminooxyalkanoic acid (no pKa's 6.0–6.5 are observable) and that the most probable sequence is the hydrolysis to hydroxylamine and some carboxylic acid, with a subsequent fast degradation of hydroxylamine to ammonia and nitrite ion.

Figure 5 plots the ammonia and nitrite produced as a function of time in 0.01 *M* sodium hydroxide at 60° as based on the above discussion. Apparent first-order plots for the production of ammonia and nitrite are given in Fig. 6 for aminooxyacetic acid with an apparent half-life of 110 hours under these conditions.

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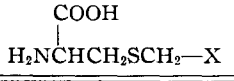
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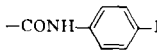
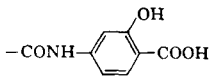
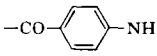
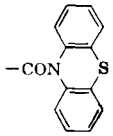
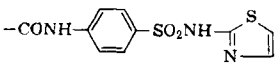
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The sulfide derivatives of cysteine, L-3-(substituted)-methylthioalanines (Table I), were made by

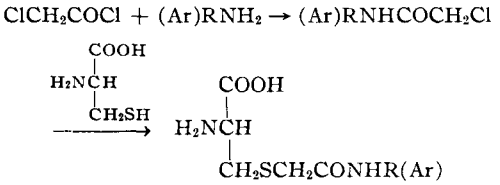
TABLE I.—L-3-(SUBSTITUTED)-METHYLTHIOALANINES



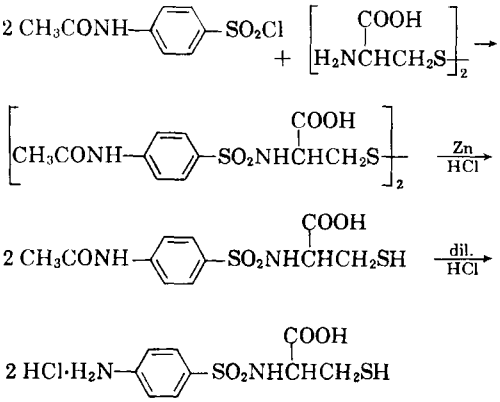
No.	X	Formula	Yield, %	M.P.,[a] °C. (decompn.)	Ref.[b]	Nitrogen, % Calcd.	Nitrogen, % Found.[c]	Sulfur, % Calcd.	Sulfur, % Found
1		$C_{13}H_{17}N_3O_4S$	38.8	208	(4)	13.50	13.32	10.30	9.88
2		$C_{12}H_{14}N_2O_6S$	18.0	203	(5)	8.92	8.90	10.20	10.18
3		$C_{11}H_{14}N_2O_3S$	60.2	130	...	11.02	10.92	12.61	12.65
4		$C_8H_{14}N_2O_3S$	34.4	183	(6)	12.84	13.01	14.69	15.02
5		$C_{17}H_{16}N_2O_3S_2$	61.9	165	(7)	7.77	7.42	17.79	17.60
6		$C_{11}H_{16}N_4O_5S_3$	10.3	164	(8)	13.45	12.99	23.10	23.32
7		$C_{14}H_{15}NO_2S$	56.5	197	...	5.36	5.19	12.27	11.51

[a] All melting points are uncorrected. [b] These references pertain to the synthesis of the corresponding N-chloroacetyl precursors made by treating those substituted amines grouped under the X column with chloroacetyl chloride. [c] The analytical data were determined at the Sterling-Winthrop Research Institute.

the alkylation of cysteine according to procedures previously described (1-3).



The new sulfonamides of cysteine and methionine (Table II) were made in the usual manner starting with N-acetylsulfanilyl chloride (ASC) to acylate the amino groups of these amino acids, followed by the acid hydrolysis of the acetamido grouping. In the case of cysteine, the acylation was first performed on cystine and the resulting product then reduced to free the mercapto group, according to the following reactions



EXPERIMENTAL

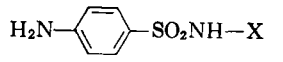
Most of the starting materials were obtained from Eastman. Others were purchased from Matheson (*p*-aminosalicylic acid and DL-methionine) and Merck (sulfathiazole).

L-3-(Substituted)-methylthioalanines (Table I).—Compounds 1 and 6 were recrystallized from water. Compound 4 was recrystallized from water and alcohol. Number 3 required two recrystallizations from water and a third from equal parts absolute ethanol and methanol, followed by the addition of ether. Number 7 was purified from acetic acid plus water. For compound 5, equal parts of alcohol and water was used initially, and then water and acetic acid. Compound 2 required alternate treatment with dilute hydrochloric acid and sodium bicarbonate solutions. The base, added to the filtered, acid solution of the crude material, yielded the amphoteric compound, which redissolved by the addition of more bicarbonate solution. Acidification of the filtered, basic solution produced a pure compound.

L - N,N' - Bis - (*p* - acetamidobenzenesulfonyl)-cystine.—A mixture consisting of 39.2 Gm. (0.168 mole) of ACS, 20.0 Gm. (0.084 mole) of L(−)cystine, 13.6 Gm. (0.336 mole) of sodium hydroxide dissolved in 500 ml. of water, and 300 ml. of ether was vigorously agitated for 45 minutes at room temperature. The layers were separated and the aqueous portion chilled and acidified with concentrated hydrochloric acid to pH 1. The product was reduced at once without further purification. For analysis, a portion was recrystallized once from isopropyl alcohol and twice from alcohol; m.p. 216° (decompn.).

Anal.—Calcd. for $C_{22}H_{26}N_4O_{10}S_4$: N, 8.83; S, 20.20. Found: N, 8.82; S, 19.6.

TABLE II.—SOME SULFONAMIDE DERIVATIVES OF CYSTEINE AND METHIONINE



No.	X	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found
8	—CH(COOH)CH$_2$SH	$C_9H_{12}N_2O_4S$	10.14	10.29	23.21	23.40
9	—CH(COOH)CH$_2$SCH$_2$CH$_3$	$C_{11}H_{16}N_2O_4S_2$	9.20	9.08	21.07	21.5
10	—C$_6$H$_4$—SO$_2$NHCH(COOH)CH$_2$SC$_2$H$_5$	$C_{17}H_{21}N_3O_6S_3$	9.14	8.75	20.93	20.03
11	—CH(COOH)CH$_2$CH$_2$SCH$_3$	$C_{11}H_{16}N_2O_4S_2$	...	[a]	21.07	22.38

[a] Carbon and hydrogen were also determined. *Anal.*—Calcd.: C, 43.40; H, 5.30. Found: C, 43.77; H, 5.17.

L - 3 - Mercapto - N - acetylsulfanilylalanine.—The crude L-N,N′-Bis-(*p*-acetamidobenzenesulfonyl)-cystine was treated with 9.0 Gm. (0.138 mole) of zinc dust, 90 ml. (0.168 mole) of a 10% solution of hydrochloric acid, 110 ml. methyl alcohol, and 75 ml. of water. The mixture was then heated at about 55° for 1.5 hours, with shaking. To effect a solution of the product, the mixture was heated to near boiling, filtered, and chilled. The white product thus obtained was essentially pure and amounted to 14.6 Gm., or 27.4%, based on the quantity of ASC used initially. A sample, recrystallized twice from alcohol, melted at 214–217°.

Anal.—Calcd. for $C_{11}H_{14}N_2O_5S_2$: N, 8.80; S, 20.14. Found: N, 8.85; S, 19.9.

L-3-Mercapto-N-sulfanilylalanine (Compound 8).—The previously isolated 14.6 Gm. (0.046 mole) of L-3-mercapto-N-acetylsulfanilylalanine was refluxed for 30 minutes with 125 ml. of 6 *N* hydrochloric acid solution. The mixture was filtered while hot, cooled, and neutralized with a solution of 20% sodium hydroxide. The 8.0 Gm. of product, obtained by two recrystallizations from hot water, corresponded to a 63% yield; m.p. 170–173°. For analytical data, see Table II.

L - 3 - Ethylthio - N - (N′ - acetylsulfanilyl)-alanine.—By the procedure previously described, a mixture of 13.4 Gm. (0.090 mole) of L-3-ethylthioalanine (2), 22.2 Gm. (0.095 mole) of ASC, 7.6 Gm. (0.190 mole) of sodium hydroxide in 150 ml. of water, and 150 ml. of ether, stirred for 2 hours at room temperature, yielded 17.4 Gm. of product, which was recrystallized twice from alcohol and water. Yield, 55.9%; m.p. 190–193°.

Anal.—Calcd. for $C_{13}H_{18}N_2O_5S_2$: N, 8.09; S, 18.51. Found: N, 8.02; S, 18.69.

L-3-Ethylthio-N-sulfanilylalanine (Compound 9).—From the hydrolysis of 17.4 Gm. (0.051 mole) of L-3-ethylthio-N-(N′-acetylsulfanilyl)-alanine with 125 ml. of 6 *N* hydrochloric acid solution for 30 minutes was obtained 11.1 Gm. of product, following two recrystallizations from alcohol and water. Yield, 70.6%; m.p. 182–184°.

L - 3 - Ethylthio - N - [(N′ - sulfanilyl) - sulfanilyl]-alanine (Compound 10).—As previously described, the crude product obtained from a mixture of 7.7 Gm. (0.025 mole) of L-3-ethylthio-N-sulfanilylalanine, 6.5 Gm. (0.028 mole) of ASC, 2.2 Gm. (0.056 mole) of sodium hydroxide in 80 ml. of water, and 80 ml. of ether, was hydrolyzed directly with 60 ml. of 6 *N* hydrochloric acid solution for one-half hour. The product thus obtained was recrystallized three times from alcohol and water, yielding 1.5 Gm. or 12%, based on L-3-ethylthio-N-sulfanilylalanine; m.p. 127° (decompn.).

DL-N-(N′-Acetylsulfanilyl)-methionine.—A mixture of 13.9 Gm. (0.093 mole) of DL-methionine, 21.6 Gm. (0.093 mole) of ASC, 6.8 Gm. (0.186 mole) of sodium hydroxide dissolved in 110 ml. of water, and 110 ml. of ether, agitated for 1 hour, yielded an oil, upon acidification of the aqueous phase. The oil solidified in a desiccator over calcium chloride after 3 days. Partial purification was effected by triturating the solid in a mortar three times with 50-ml. portions of ether, the mixture being filtered each time. The undissolved substance was then recrystallized once from ethyl acetate and again from water. The 12.0 Gm. of product thus obtained corresponded to a 37.6% yield; m.p. 163–164°.

Anal.—Calcd. for $C_{13}H_{18}N_2O_5S_2$: C, 45.07; H, 5.24; N, 8.09; S, 18.51. Found: C, 44.98; H, 4.97; N, 7.92; S, 18.67.

DL-N-Sulfanilylmethionine (Compound 11).—Hydrolysis of 12.0 Gm. (0.035 mole) of DL-N-(N′-acetylsulfanilyl)-methionine with 95 ml. of 6 *N* hydrochloric acid solution in the usual way produced 4.2 Gm. of product (39.8%), after two recrystallizations from ethyl acetate; m.p. –144146°.

BIOLOGICAL RESULTS[1]

Compounds 2, 5, 7, 8, and 10 were ineffective intraperitoneally or subcutaneously against a *M. pyogenes* infection in Swiss mice. Also, compounds 8, 9, 10, and 11 were inactive *in vivo* against a staphylococcus infection orally and subcutaneously. Against a *Trichomonas gallinae* infection in hamsters, compound 7 was without effect.

[1] The author wishes to express his thanks to A. J. Arrowsmith, S. D. Sobell, D. A. Berberian, and R. G. Slighter, among others, of the Sterling-Winthrop Research Institute for performing the biological studies on these compounds.

As possible antimetabolites for calcium pantothenate, phenylalanine, cysteine, and folic acid, compounds 3, 4, 5, 6, and 11 were ineffective. Given intraperitoneally to Swiss mice having a *D. pneumoniae I* infection, compounds 2, 3, 4, and 6 were of no value. Compound 1 was ineffective as a possible spermatocidal agent. Also ineffective against a *Klebsiella pneumoniae* infection in Swiss mice given by various routes were compounds 1, 3, 4, 5, and 6. Compound 2, however, did show slight effectiveness.

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Effect of Phentolamine and Tolazoline on Some Metabolic Processes of Heart Homogenates

BY HARRY C. FROEDE and HAROLD C. HEIM

The addition of phentolamine enhances oxygen uptake of rat heart homogenates respiring in the presence of added succinate, but evokes an inhibitory effect with pyruvate as the substrate. These effects are not observed when phentolamine is replaced by tolazoline. Oxidation of α-ketoglutarate is not affected by either drug nor is the activity of a purified malic dehydrogenase. The addition of oxaloacetate to homogenates inhibits oxygen uptake and the inhibitory effect is not abolished by the prior addition of phentolamine. The total α-keto acid content of homogenates respiring in the presence of succinate was found to be somewhat higher than in those homogenates to which both succinate and phentolamine were added at the beginning of the experiments.

TOLAZOLINE and phentolamine are both 2-substituted imidazolines which evoke adrenergic blocking effects. It has been reported that the most pronounced side effects elicited by these drugs are related to a direct stimulation of the myocardium and include cardiac arrhythmias, anginal pain, tachycardia, and fibrillation (1–4).

A survey of the literature reveals that no studies have been undertaken to determine whether phentolamine or tolazoline affect metabolic processes of the heart, *in vitro*. Therefore, because of the widely held belief that drugs produce effects on tissues, at least in part, through an alteration of intracellular reactions, an investigation of the effects of these two drugs on oxidative processes of heart homogenates was conducted.

EXPERIMENTAL

Conventional manometric techniques were used to determine the effect of phentolamine and tolazoline on oxygen uptake in the presence of different added substrates including succinate, α-ketoglutarate, and pyruvate. Hearts from adult Sprague-Dawley rats of both sexes were used. The animals were stunned and decapitated, following which the hearts were immediately removed, cut open, and washed in cold 0.1*M* phosphate buffer. The ventricles were then dissected free, blotted dry on filter paper, weighed, and placed in a glass homogenizer immersed in ice and containing sufficient buffer so that 1.0 ml. of homogenate contained 50 mg., wet weight, of ventricle. The homogenates were centrifuged for 10 minutes at 480 × g in a Servall RC-2 centrifuge with an SS-34 rotor and at a temperature of 3°. The sediment was discarded and the supernatant was used as the homogenate. Ten animals were used in each experiment and each determination of oxygen uptake was performed in duplicate. No more than 10 minutes elapsed between the time the animals were killed and the preparation of the homogenates.

The concentrations of reagents added to the flasks are included in *Results*. The temperature of the bath was 37° and the gas phase was air. After a 10-minute equilibration period, the side arm contents were tipped into the vessels, the manometers closed, and readings taken at 15-minute intervals for a total of 90 minutes.

Keto-acid determinations were performed according to the method of Friedemann (5). The contents of the main compartments of two Warburg vessels were pooled and 2.0 ml. of the ensuing mixture was pipetted into 8.0 ml. of cold, 10% trichloroacetic acid solution. After centrifugation at 3000 × g for 15 minutes, 3.0 ml. of the clear supernatant

Received September 6, 1961, from the University of Colorado, School of Pharmacy, Boulder.
Accepted for publication October 6, 1961.
This investigation was supported by grant number H-5476 from the National Institutes of Health, United States Public Health Service.
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Effect of Phentolamine and Tolazoline on Some Metabolic Processes of Heart Homogenates

BY HARRY C. FROEDE and HAROLD C. HEIM

The addition of phentolamine enhances oxygen uptake of rat heart homogenates respiring in the presence of added succinate, but evokes an inhibitory effect with pyruvate as the substrate. These effects are not observed when phentolamine is replaced by tolazoline. Oxidation of α-ketoglutarate is not affected by either drug nor is the activity of a purified malic dehydrogenase. The addition of oxaloacetate to homogenates inhibits oxygen uptake and the inhibitory effect is not abolished by the prior addition of phentolamine. The total α-keto acid content of homogenates respiring in the presence of succinate was found to be somewhat higher than in those homogenates to which both succinate and phentolamine were added at the beginning of the experiments.

TOLAZOLINE and phentolamine are both 2-substituted imidazolines which evoke adrenergic blocking effects. It has been reported that the most pronounced side effects elicited by these drugs are related to a direct stimulation of the myocardium and include cardiac arrhythmias, anginal pain, tachycardia, and fibrillation (1–4).

A survey of the literature reveals that no studies have been undertaken to determine whether phentolamine or tolazoline affect metabolic processes of the heart, *in vitro*. Therefore, because of the widely held belief that drugs produce effects on tissues, at least in part, through an alteration of intracellular reactions, an investigation of the effects of these two drugs on oxidative processes of heart homogenates was conducted.

EXPERIMENTAL

Conventional manometric techniques were used to determine the effect of phentolamine and tolazoline on oxygen uptake in the presence of different added substrates including succinate, α-ketoglutarate, and pyruvate. Hearts from adult Sprague-Dawley rats of both sexes were used. The animals were stunned and decapitated, following which the hearts were immediately removed, cut open, and washed in cold 0.1*M* phosphate buffer. The ventricles were then dissected free, blotted dry on filter paper, weighed, and placed in a glass homogenizer immersed in ice and containing sufficient buffer so that 1.0 ml. of homogenate contained 50 mg., wet weight, of ventricle. The homogenates were centrifuged for 10 minutes at 480 × g in a Servall RC-2 centrifuge with an SS-34 rotor and at a temperature of 3°. The sediment was discarded and the supernatant was used as the homogenate. Ten animals were used in each experiment and each determination of oxygen uptake was performed in duplicate. No more than 10 minutes elapsed between the time the animals were killed and the preparation of the homogenates.

The concentrations of reagents added to the flasks are included in *Results*. The temperature of the bath was 37° and the gas phase was air. After a 10-minute equilibration period, the side arm contents were tipped into the vessels, the manometers closed, and readings taken at 15-minute intervals for a total of 90 minutes.

Keto-acid determinations were performed according to the method of Friedemann (5). The contents of the main compartments of two Warburg vessels were pooled and 2.0 ml. of the ensuing mixture was pipetted into 8.0 ml. of cold, 10% trichloroacetic acid solution. After centrifugation at 3000 × g for 15 minutes, 3.0 ml. of the clear supernatant

Received September 6, 1961, from the University of Colorado, School of Pharmacy, Boulder.
Accepted for publication October 6, 1961.
This investigation was supported by grant number H-5476 from the National Institutes of Health, United States Public Health Service.
The authors are most grateful for the generous amounts of phentolamine and tolazoline supplied by the Ciba Pharmaceutical Co.

was used for determination of total keto-acid content. The color which developed upon addition of 2,4-dinitrophenylhydrazine was measured at 435 mμ, with redistilled pyruvic acid being used as the standard keto-acid.

Malic dehydrogenase activity was determined according to the method of Ochoa (6). The oxidation of dihydro-β-diphosphopyridine nucleotide (DPNH) to diphosphopyridine nucleotide (DPN), with the concomitant reduction of oxaloacetate to malate, was measured by observing the decrease in absorbance (A_s) at 340 mμ with a Beckman model DU spectrophotometer. The enzyme concentration was adjusted so that 3.0 ml. of reaction mixture contained 40 units. The final concentration of DPNH and oxaloacetate were both 5×10^{-6} *M*. Malic dehydrogenase, DPNH, and oxaloacetate solutions were freshly prepared prior to each experiment.

RESULTS

Figure 1 shows that the oxygen consumption by rat heart homogenates in the presence of added succinate proceeds at a rapid rate for approximately 30 minutes, after which a decline in rate occurs. This is confirmatory to the results obtained by other investigators (7, 8). Figure 1 also shows, however, that if phentolamine is added at the start of the experiment in sufficient amount so that the final concentration is 7×10^{-4} *M*, oxygen consumption is much greater than in the absence of phentolamine. The rate is initially quite rapid and declines somewhat after about 30 minutes, but still remains greater than in the control flasks. The prior addition of tolazoline, so that final concentrations ranged between 1.4×10^{-2} *M* and 3.6×10^{-4} *M*, did not significantly alter the rate of oxygen uptake.

It has been established that oxaloacetate markedly inhibits the oxidation of succinate (9, 10) and it has been suggested that the decline in rate of oxygen uptake might be due to accumulation of oxaloacetate (7). Table I shows that the addition of oxaloacetate inhibited oxygen uptake in the presence of succinate by approximately 57% and that this inhibitory effect was not significantly altered by either phentolamine or tolazoline. In flasks containing pyruvate as the substrate, the addition of phentolamine, at final concentration of 7×10^{-4} *M*, caused a 70% inhibition of oxygen uptake, but this effect was not observed with tolazoline. When malate and succinate were both present, the oxygen uptake was somewhat less than that obtained when no malate was present, and the addition of phentolamine caused a 210% increase in oxygen consumption. Similar results were obtained with phentolamine in the experiments in which fumarate was present together with succinate. Tolazoline did not significantly alter oxygen consumption. When α-ketoglutarate was added as the substrate, the oxygen consumption was not changed by either phentolamine or tolazoline.

Analysis of flask contents at the end of the experiments revealed that total α-keto acids were present

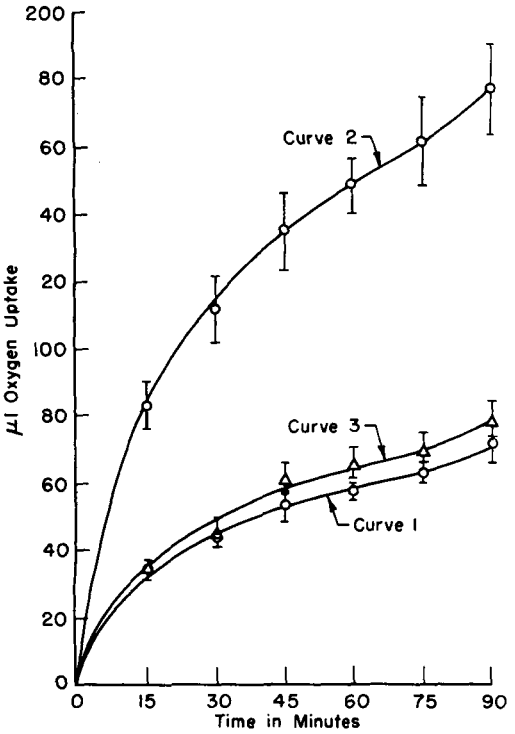


Fig. 1.—Effect of phentolamine and tolazoline on rate of oxygen uptake in the presence of added succinate. Curve 1, no drug; curve 2, phentolamine (7×10^{-4} *M*); curve 3, tolazoline (7×10^{-4} *M*). Experimental conditions are the same as shown in Table I.

TABLE I.—EFFECT OF PHENTOLAMINE AND TOLAZOLINE ON OXYGEN UPTAKE

Substrate	Drug	O_2 Uptake, mm.³/25 mg. Tissue/90 min.	Increase (+) or Decrease (−) Due to Drug, %
Succinate[a]	None	72 ± 5.7[g]	
	Phentolamine	176 ± 12.6	+140
	Tolazoline	77 ± 5.1	...
Pyruvate[b]	None	110 ± 7.9	
	Phentolamine	34 ± 6.0	−69
	Tolazoline	85 ± 9.7	−23
Succinate + malate[c]	None	54 ± 8.7	
	Phentolamine	165 ± 8.9	+210
	Tolazoline	58 ± 8.1	...
Succinate + fumarate[d]	None	41 ± 4.4	
	Phentolamine	132 ± 15.3	+280
	Tolazoline	47 ± 4.9	...
Succinate + oxaloacetate[e]	None	31 ± 5.5	
	Phentolamine	40 ± 7.7	...
	Tolazoline	34 ± 4.2	...
α-Keto-glutarate[f]	None	31 ± 4.4	
	Phentolamine	32 ± 4.1	...
	Tolazoline	31 ± 5.0	...

[a] Flasks contained 0.4 ml. 0.1 *M* succinate tipped from sidearm. [b] Flasks contained 0.4 ml. 0.1 *M* pyruvate containing 7.5×10^{-3} *M* ATP, 2.5×10^{-4} *M* DPN, and 2.5×10^{-3} *M* $MgCl_2$ added from sidearm. Main compartment contained 0.1 ml. 1.5×10^{-2} *M* nicotinamide. [c] Flasks contained 0.4 ml. 0.1 *M* succinate and 0.2 ml. 2×10^{-2} *M* malate, both added from sidearm. [d] Flasks contained 0.4 ml. 0.1 *M* succinate and 0.2 ml. 2×10^{-2} *M* fumarate, both added from sidearm. [e] Flasks contained 0.4 ml. 0.1 *M* succinate in sidearm, 0.1 ml. 10^{-2} *M* oxaloacetate in main compartment. [f] Flasks contained 0.4 ml. 0.1 *M* α-ketoglutarate and 0.1 ml. 10^{-3} *M* DPN, both added from sidearm. In addition, all flasks contained 0.5 ml. homogenate (25 mg. tissue), 0.3 ml. 5×10^{-4} *M* cytochrome C, 1.0 ml. 0.1 *M* phosphate buffer, pH 7.4 and water to make a total volume of 3.0 ml. Center wells contained 0.2 ml. 10% potassium hydroxide and filter paper strips. [g] Standard deviation = $\sqrt{\frac{\Sigma d^2}{n-1}}$.

TABLE II.—TOTAL KETO ACIDS IN FLASK CONTENTS AFTER 90 MINUTES

Substrate	Keto Acids, mcg./25 mg. Tissue[a]
Succinate	46.0 ± 2.2
Succinate + phentolamine	31.6 ± 3.7

[a] Results represent average of six determinations.

in greater amounts in flasks containing succinate than in those containing succinate plus phentolamine. These results are set forth in Table II.

Neither phentolamine nor tolazoline produced any significant effect on the activity of a purified malic dehydrogenase obtained from commercial sources.[1]

DISCUSSION

Although phentolamine and tolazoline are related chemically, tolazoline did not produce the *in vitro* metabolic effects observed with phentolamine. A slight inhibition of pyruvate oxidation was observed with tolazoline, but the effect was not nearly so marked as that observed with phentolamine.

Several investigators (7, 9, 10) have reported that very low concentrations of oxaloacetate strongly inhibit the oxidation of succinate. In this study it was found that the inhibitory effect of oxaloacetate could not be abolished by the prior addition of phentolamine. It has been suggested that oxaloacetate inhibits the oxidation of succinate through a competitive inhibition of succinic dehydrogenase (9–11) and at least one study has been conducted in which this phenomenon was observed with a purified succinic dehydrogenase preparation (12). It seems likely, however, that mass action effects might account for some of the inhibition when homogenates are used as the enzyme source because such preparations contain many enzymes other than succinic dehydrogenase and which could conceivably be affected by oxaloacetate. For example, the oxidation of succinate proceeds according to the pathway

$$\text{succinate} \xrightarrow{\text{DPN}} \text{fumarate} \rightarrow \text{malate} \xrightarrow{\text{DPN}} \text{oxaloacetate}.$$

The equilibrium constant (K') for the fumarate-malate system has been reported to be $8 \times 10^{-13}\ M$ (13). In view of the fact that the equilibrium lies far in the direction of malate, only minute amounts of oxaloacetate would be necessary to stop the conversion of malate to oxaloacetate.

The α-keto acid content, after 90 minutes incubation with succinate, was reduced by about 33% in those flasks to which phentolamine had been added at the beginning of the experiment. Wolff and Ball (7), in their studies on the effect of thyroxine on succinate metabolism, found that thyroxine inhibited malic dehydrogenase and concluded that thyroxine prevented the accumulation of oxaloacetate. The concentration of succinate used in these studies was much greater than that used by Wolff and Ball. Thus, if oxaloacetate is a competitive inhibitor of succinic dehydrogenase, a greater quantity would be required to inhibit oxygen uptake in the experiments reported herein. It was found, however, that the total amount of α-keto acids which accumulated in the flask contents is in close agreement with the values reported by Wolff and Ball. Furthermore, the rate of oxygen uptake during the first 15 minutes was about 140% greater in the flasks containing phentolamine, whereas thyroxine has been reported to produce no change in the initial rate (7). It would not appear conceivable, therefore, that phentolamine increases the oxygen uptake in the presence of added succinate by preventing the accumulation of oxaloacetate.

Close correlation cannot as yet be drawn between the effects observed *in vitro* and the response elicited by drugs *in vivo*. It has been established that even slight alterations in the structural configuration of many drugs produce marked changes in pharmacological activity. Although phentolamine and tolazoline are both imidazoline derivatives, they differ structurally to a considerable extent. This structural difference could possibly be related to the different effects produced by the two drugs on the *in vitro* systems used in this study and to the effects produced on the heart, *in vivo*.

It should be stressed that, with the exception of the experiments involving purified malic dehydrogenase, this study was conducted with homogenates as the enzyme source. In such preparations there are many reactions occurring which evoke indirect, rather than direct, effects on the enzymatic reactions of the citric acid cycle. In describing the effects of a drug on aerobic processes one cannot neglect the possibility that the drug might alter reactions which are indirectly involved with the cycle and which, therefore, escape observation when studies are performed using citric acid cycle intermediates as substrates. It is, therefore, not possible to elaborate a mechanism of action of phentolamine on the basis of the effects produced by this drug on enzymes of the citric acid cycle, *in vitro*. However, since phentolamine inhibits oxygen uptake with pyruvate as substrate, enhances oxygen uptake in the presence of succinate, evokes no effect on α-ketoglutarate oxidation, and has no effect on malic dehydrogenase, it could conceivably act at the level of the electron transport system or the accompanying phosphorylation system.

[1] Sigma Chemical Corp., St. Louis, Mo.

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Indanols V

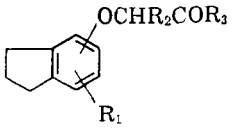
Indanoxyacetic Acid Derivatives

By SEYMOUR L. SHAPIRO†, THEODORE BAZGA, and LOUIS FREEDMAN

Indanoxyacetic acid derivatives (I) yielded compounds with adrenergic blocking and potentiating effects, tranquilizing activity, and anti-inflammatory activity. Of particular interest was the hypocholesteremic effect with α-(indan-4-oxy)butyric acid.

In the preceding paper, indanoxy ethers (1) were projected as simple structural analogs (2, 3) of steroids and related compounds.

Herein, we evaluate indanoxyacetic acid analogs of the type I



I

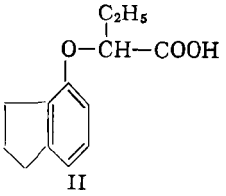
which were varied as shown in Table I. Such structures show formal analogies to the α-biphenylbutyric acids evaluated as hypocholesteremic agents by Garattini and co-workers (4), as well as the auxins (5).

Group variation, as for example R_1 as benzyl and *p*-chlorobenzyl, provided enhanced lipid solubility (6) whereas employment of R_2 from hydrogen through *n*-butyl assessed structures with increasing molecular crowding about the carboxyl group (7). The R_3 group, in turn, was inspected as the indanoxyacetic acids and derived amides and dialkylaminoalkylamides.

Reaction of the appropriate indanol with the substituted ethyl-α-bromoacetate in refluxing acetone (8), employing anhydrous potassium carbonate as an acid binder, gave the ester I, $R_3 = OC_2H_5$. The esters were hydrolyzed to the corresponding acids or converted to the amides.

On oral administration, cholesterolytic activity (8) was noted as follows: compound No./$LD_{min.}$ mg./Kg. s.c./oral dosage mg./Kg., for 3 days/% reduction in serum cholesterol at end of three days: 54/750/250/37; 55/1000/200/31; 56/300/100/51; 57/500/166/none; 58/450/150/none; 12/750/250/12. The 5-position isomer of compound 55, compound 14 ($LD_{min.}$ 500 mg./Kg.) showed 18% reduction in cholesterol after three daily doses at 30 mg./Kg. s.c. Compound 54 afforded lasting hypotension whereas compounds 55 and 56 were without effect on blood pressure (9).

The high order of hypocholesteremia obtainable with compound 56 (II), in the absence of hypotensive activity



II

has suggested additional explorations associated with the steric factor at R_2, along with the influence of substituents *ortho* to the phenoxy oxygen.

Other pharmacological effects of interest were noted as follows: potentiation of adrenaline (compounds 21, 22, 25, 31, and 39), and adrenergic block (compounds 68 and 69); ganglionic block (compound 68); reduction in motor activity (compounds 23, 25, and 72); significant inhibition of mescaline scratch test, with no inhibition of motor activity (compounds 29, 69, and 76); anti-inflammatory effects (compounds 31 and 77).

EXPERIMENTAL[1]

Ethyl α-(Indan-4-oxy)butyrate. (Compound 44).—A mixture of 26.8 Gm. (0.2 mole) of 4-indanol, 39 Gm. (0.2 mole) of ethyl α-bromobutyrate, and 27.6 Gm. (0.2 mole) of anhydrous potassium carbonate in 80 ml. of acetone was stirred and heated under reflux for 4 hours. When cool, the solid was removed by filtration and the filtrate diluted with 250 ml. of ice water and extracted with three 100-ml. portions of ether. The ethereal extracts were combined, washed successively with *N* sodium hydroxide and water, and then dried (anhydrous magnesium sulfate). After filtration, the ether was removed and the residue distilled to give 24 Gm. (48%) of product, b.p. 114–118° at 0.2 mm.

N - Diethylaminoethyl - α - (indan - 4 - oxy)-butyric Acid Amide. (Compound 69.)—A mixture

[1] Typical experimental procedures are given.

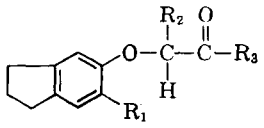
Received September 18, 1961, from the Research Laboratories, U. S. Vitamin & Pharmaceutical Corp., 26 Vark St., Yonkers 1, N. Y.

Accepted for publication October 5, 1961.

The authors are grateful to Dr. G. Ungar and his staff for the pharmacological results herein reported.

† Deceased.

TABLE I.—INDANOXYACETIC ACID DERIVATIVES



No.	R_1	R_2	M.p., °C.[a] B.p., °C. (mm. Press.)	Formula	Analysis, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
				R_3 = —OC_2H_5[b]						
1	H	H	118–120 (0.15)	$C_{13}H_{16}O_3$	70.9	70.8	7.3	8.0	...	...
2	H	CH_3–	110–116 (0.25)	$C_{14}H_{18}O_3$	71.8	72.2	7.7	7.9	...	...
3	H	C_2H_5—	104–110 (0.09)	$C_{15}H_{20}O_3$	72.6	73.1	8.1	8.3	...	...
4[c]	H	*n*-C_3H_7—	134 (0.09)	$C_{16}H_{20}O_3$	72.6	72.7	8.1	8.2	...	...
5	H	*i*-C_3H_7—	110–116 (0.1)		...	...	...	...	...	...
6	$C_6H_5CH_2$—	H	168–178 (0.1)	$C_{20}H_{22}O_3$	77.4	77.4	7.1	7.0	...	...
7	$C_6H_5CH_2$—	CH_3	164–168 (0.4)		...	...	...	...	...	...
8	$C_6H_5CH_2$—	C_2H_5—	170–180 (0.5)		...	...	...	...	...	...
9	*p*-$ClC_6H_4CH_2$—	H	188–194 (0.16)		...	...	...	...	...	...
10	*p*-$ClC_6H_4CH_2$—	CH_3—	176–182 (0.2)	$C_{21}H_{23}ClO_3$	70.3	70.4	6.4	6.5	...	...
11	*p*-$ClC_6H_4CH_2$—	C_2H_5—	184–190 (0.05)		...	...	...	...	...	...
				R_3 = —OH						
12[d]	H	H	159–160 (C)	$C_{11}H_{12}O_3$	68.7	69.1	6.3	6.4	...	...
14	H	C_2H_5—	84–85 (A)	$C_{13}H_{16}O_3$	70.9	71.0	7.3	7.4	...	...
15	H	*n*-C_3H_7—	71–72 (A)	$C_{14}H_{18}O_3$	71.8	72.0	7.7	7.8	...	...
16	H	*i*-C_3H_7—	88–90 (A)	$C_{14}H_{18}O_3$	71.8	71.6	7.7	7.5	...	...
17	*p*-$ClC_6H_4CH_2$—	H	135–136 (B)	$C_{18}H_{17}ClO_3$	68.2	68.3	5.4	5.5	11.2[e]	11.2
18	*p*-$ClC_6H_4CH_2$—	CH_3	125–128 (C)	$C_{19}H_{19}ClO_3$	68.9	68.8	5.7	6.0	10.7[e]	10.6
19	*p*-$ClC_6H_4CH_2$—	C_2H_5—	155–157 (A)	$C_{20}H_{21}ClO_3$	69.6	70.0	6.1	5.9	10.2[e]	10.4
				R_3 = —$NHCH_2CH_2C_6H_5$						
20	H	H	97–98 (B)	$C_{19}H_{21}NO_2$	77.3	77.6	7.2	7.2	4.7	4.5
21	H	CH_3—	62–63 (A)	$C_{20}H_{23}NO_2$	77.6	77.7	7.5	7.3	...	...
22	H	C_2H_5—	64 (B)	$C_{21}H_{25}NO_2$	78.0	78.2	7.8	7.3	4.3	4.2
23	$C_6H_5CH_2$—	C_2H_5—	208–214 (0.04)	$C_{28}H_{31}NO_2$	...	...	...	...	3.4	3.3
24	*p*-$ClC_6H_4CH_2$—	C_2H_5—	85–90 (D)	$C_{28}H_{30}ClNO_2$	75.1	75.1	6.8	6.9	3.1	3.2
				R_3 = —$NHCHCH_3CH_2C_6H_5$[f]						
25	H	CH_3—	121–122 (B)	$C_{21}H_{25}NO_2$	78.0	77.9	7.8	7.9	4.3	4.3
26	H	C_2H_5—	130–131 (B)	$C_{22}H_{27}NO_2$	78.3	78.4	8.1	7.9	4.2	4.0
27	$C_6H_5CH_2$—	C_2H_5—	234–244 (0.2)	$C_{29}H_{33}NO_2$	81.5	81.7	7.8	7.3	...	...
				R_3 = —$NH(CH_2)_2N(C_2H_5)_2$						
28	H	H	166–176 (0.25)	$C_{17}H_{26}N_2O_2$	70.3	70.0	9.0	9.0	9.6	9.5
29	H	CH_3—	160–166 (0.08)	$C_{18}H_{28}N_2O_2$	71.0	70.6	9.3	9.2	9.2	9.5
30	H	C_2H_5—	166 (0.28)	$C_{19}H_{30}N_2O_2$	...	...	...	...	8.8	9.0
31	$C_6H_5CH_2$—	C_2H_5—	202–212 (0.12)	$C_{26}H_{36}N_2O_2$	76.4	76.5	8.9	8.8	6.9	6.7
32	*p*-$ClC_6H_4CH_2$—	C_2H_5—	224 (0.05)	$C_{26}H_{35}ClN_2O_2$	70.4	71.0	7.9	7.9	...	...
				R_3 = —$NH(CH_2)_3N(CH_3)_2$						
33	H	H	178–180 (0.46)	$C_{16}H_{24}N_2O_2$	69.5	69.9	8.7	8.9	...	...
34[g]	H	H	120–122 (E)	$C_{22}H_{27}N_5O_9$	52.3	52.3	5.4	5.3	...	...
35	H	C_2H_5—	160–164 (0.02)	$C_{18}H_{28}N_2O_2$	71.0	70.8	9.3	9.2	9.2	9.1
36	*p*-$ClC_6H_4CH_2$	C_2H_5—	226 (0.09)	$C_{26}H_{33}ClN_2O_2$	70.0	69.9	7.8	7.9	...	...
				R_3 = —$NH(CH_2)_3N(C_2H_5)_2$						
37	H	CH_3—	164–176 (0.11)	$C_{19}H_{30}N_2O_2$	...	...	...	...	8.8	9.2
38[g]	H	CH_3—	101–103 (E)	$C_{25}H_{32}N_6O_9$	54.8	54.5	6.1	6.0	...	...
39	H	C_2H_5—	172 (0.03)	$C_{20}H_{32}N_2O_2$	72.3	72.6	9.7	9.7	8.4	8.3
40	$C_6H_5CH_2$—	C_2H_5—	212–216 (0.06)	$C_{27}H_{38}N_2O_2$	...	...	...	...	6.6	6.3
41	*p*-$ClC_6H_4CH_2$—	C_2H_5—	216–218 (0.05)	$C_{27}H_{37}ClN_2O_2$	70.9	71.0	8.2	7.9	...	...
				R_3 = —OC_2H_5[b]						
42	H	H	112–116 (0.28)	$C_{13}H_{16}O_3$	70.9	70.9	7.3	7.6	...	...
43	H	CH_3—	104–110 (0.18)	$C_{14}H_{18}O_3$	71.8	72.3	7.7	7.9	...	...
44	H	C_2H_5—	114–118 (0.2)	$C_{15}H_{20}O_3$	72.6	73.3	8.1	8.3	...	...
45[c]	H	*n*-C_3H_7—	132 (0.4)	$C_{16}H_{20}O_3$	72.6	73.4	8.1	8.1	...	...
46	H	*i*-C_3H_7—	120 (0.1)	$C_{16}H_{22}O_3$	73.3	73.8	8.5	8.1	...	...
47	H	*n*-C_4H_9—	154 (0.3)	$C_{17}H_{24}O_3$	73.9	73.9	8.8	8.6	...	...
48	$C_6H_5CH_2$—	H	182–192 (0.03)		...	...	...	...	...	...
49	$C_6H_5CH_2$—	CH_3—	163–170 (0.19)	$C_{21}H_{24}O_3$	77.8	78.2	7.5	7.4	...	...
50	$C_6H_5CH_2$—	C_2H_5—	178–190 (0.3)		...	...	...	...	...	...
51	*p*-$ClC_6H_4CH_2$—	H	172–180 (0.08)		...	...	...	...	...	...
52	*p*-$ClC_6H_4CH_2$—	CH_3—	178–181 (0.2)		...	...	...	...	...	...
53	*p*-$ClC_6H_4CH_2$—	C_2H_5—	190–198 (0.3)		...	...	...	...	...	...
				R_3 = —OH						
54[h]	H	H	187–188 (C)	$C_{11}H_{12}O_3$	68.7	68.9	6.3	6.0	...	...
55	H	CH_3—	105–106 (A)	$C_{12}H_{14}O_3$	69.9	69.6	6.8	7.0	...	...
56	H	C_2H_5—	111–112 (A)	$C_{13}H_{16}O_3$	70.9	70.8	7.3	7.5	...	...
57	H	*n*-C_3H_7—	107–108 (A)	$C_{14}H_{18}O_3$	71.7	71.7	7.7	7.9	...	...
58	H	*n*-C_4H_9—	108–109 (A)	$C_{15}H_{20}O_3$	72.6	72.7	8.1	8.0	...	...
59	$C_6H_5CH_2$—	CH_3—	91–92 (A)	$C_{19}H_{20}O_3$	77.0	77.3	6.8	6.9	...	...
60	*p*-$ClC_6H_4CH_2$—	CH_3—	102–104 (A)	$C_{19}H_{19}ClO_3$	68.9	69.1	5.7	6.0	10.7[e]	11.1
61	*p*-$ClC_6H_4CH_2$—	C_2H_5—	116–117 (A)	$C_{20}H_{21}ClO_3$	69.6	69.4	6.1	6.3	10.3[e]	10.2
				R_3 = —$NHCH_2CH_2C_6H_5$						
62	H	H	82–84 (F)	$C_{19}H_{21}NO_2$	77.3	77.0	7.2	7.3	4.7	4.9
63	H	CH_3—	75–76 (B)	$C_{20}H_{23}NO_2$	77.6	77.9	7.5	7.6	4.5	4.6
64	H	C_2H_5—	77–79 (B)	$C_{21}H_{25}NO_2$	78.0	77.6	7.8	7.7	...	...
				R_3 = —$NHCHCH_3CH_2C_6H_5$[f]						
65	H	CH_3—	180–182 (0.18)	$C_{21}H_{25}NO_2$	78.0	78.2	7.8	8.2	4.3	4.2
66	H	C_2H_5—	136–137 (B)	$C_{22}H_{27}NO_2$	78.3	78.7	8.1	7.8	4.2	4.2

TABLE I.—(*continued*)

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69	H	C_2H_5—	156–158 (0.12)	$C_{19}H_{30}N_2O_2$	...	...	...	...	8.8	9.0
70	$C_6H_5CH_2$—	H	200–208 (0.08)	$C_{24}H_{32}N_2O_2$	75.8	75.9	8.5	8.4	7.4	7.4
71	$C_6H_5CH_2$—	C_2H_5—	204–210 (0.1)	$C_{26}H_{36}N_2O_2$	76.4	75.9	8.9	8.8	6.9	6.5
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72	H	C_2H_5—	172 (1.0)	$C_{18}H_{28}N_2O_2$	71.0	71.1	9.3	9.4	...	...
73	*p*-$ClC_6H_4CH_2$—	C_2H_5—	226 (0.09)	$C_{26}H_{33}ClN_2O_2$	70.0	69.9	7.8	7.9	...	...
				R_3 = —$NH(CH_2)_3N(C_2H_5)_2$						
74	H	H	170–174 (0.12)	$C_{18}H_{28}N_2O_2$	71.0	71.4	9.3	9.5	9.2	9.0
75	H	CH_3—	184 (0.6)	$C_{19}H_{30}N_2O_2$	...	...	...	...	8.8	8.4
76	H	C_2H_5—	176–180 (0.18)	$C_{20}H_{32}N_2O_2$	72.3	72.2	9.7	9.6	8.4	8.5
77	$C_6H_5CH_2$—	C_2H_5—	224 (0.18)	$C_{27}H_{38}N_2O_2$	...	...	...	...	6.6	6.6
78	*p*-$ClC_6H_4CH_2$—	C_2H_5—	240–250 (0.18)	$C_{27}H_{37}ClN_2O_2$	70.9	70.9	8.2	8.0	...	...

[a] Melting points are not corrected. Recrystallizing solvents: A, hexane; B, ethanol-water; C, ethanol; D, methanol; E, water; F, acetonitrile. [b] The esters in some instances were used directly without analyses. Average yield was about 50%. [c] Compound is methyl ester. [d] Reported Koelsch, C. F., and Scheiderbauer, R. A., *J. Am. Chem. Soc.*, **65**, 2311(1943), m.p. 154–155°. [e] Chlorine analysis. [f] Derived from *d*-α-methylphenethylamine. [g] Picrate of preceding compound. [h] Reported Kruber, O., and Schmieden, W., *Ber.*, **72B**, 653(1939); m.p. 182°.

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Investigations into the mechanism and kinetics of melting and crystallization of the stable form of methyl stearate as followed by changes in its specific volume have shown that: (*a*) the melting behavior was a sensitive function of its immediate history in a manner not associated with polymorphic transitions, (*b*) the thermodynamic equilibrium point was apparently displaced by stirring and that this displacement was proportional to the rate of stirring, (*c*) the rate of crystal growth for the present system was governed largely by the intrinsic rate of two-dimensional nucleation of a crystal plane, and (*d*) the rate of melting was a function of heat transport at higher temperature potentials. The extreme sensitivity of the method used allows the measurement of phase changes where they were of the order of parts per million and enabled the study under very small temperature potentials.

THE RESULTS of an investigation on the rates of melting and freezing are presented. These changes are often of great fundamental importance in altering the appearance of some and the rates of release of other pharmaceutical dosage forms. Although they are extremely common and apparently simple phenomena, relatively little effort seems to have been directed towards the construction of a clear and comprehensive picture of the underlying principles. Past studies in this area have been largely confined to metallic organic or simple organic systems which involve comparatively small entropy changes upon melt-

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THE RESULTS of an investigation on the rates of melting and freezing are presented. These changes are often of great fundamental importance in altering the appearance of some and the rates of release of other pharmaceutical dosage forms. Although they are extremely common and apparently simple phenomena, relatively little effort seems to have been directed towards the construction of a clear and comprehensive picture of the underlying principles. Past studies in this area have been largely confined to metallic organic or simple organic systems which involve comparatively small entropy changes upon melt-

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ing. The present study, on the other hand, has been concerned with the transition rates of systems which are composed of relatively large organic molecules possessing a greater entropy of melting.

The present investigation has dealt specifically with the solidifying and melting rates of methyl stearate as followed by measuring changes in its specific volume. On freezing, the ester exhibits a substantial change in volume corresponding to 30 ml. per mole, and permits the detection of an extremely small phase conversion, changes of the order of 2 p.p.m. being easily followed. These studies include (*a*) the influence of a thermal gradient on the rate of crystal growth, (*b*) melting characteristics of methyl stearate crystals formed under a moderate and extremely small temperature gradient, (*c*) the influence of a thermal gradient on the rate of melting, and (*d*) the apparent influence of stirring on the crystallization rate.

FACTORS INFLUENCING CRYSTAL GROWTH RATE

The growth of crystals involves several steps among which are transport of material to the surface, plane nucleation, growth of plane nuclei, and abstraction of the heat evolved. The overall net rate, it is evident, is dependent on the relative rates of the individual processes, the rate determining step being the slowest process. During the crystallization process, material from the melt at the interface is deposited upon the crystal and this requires, of necessity, that material must be transported from the bulk of the melt to the surface. The transport of impurity away from the surface of the crystal is also very important. If the rate of crystallization occurs at a slow rate, the concentration of impurity at the interface (molten zone) and the bulk of the melt will not appreciably differ. In the event crystallization is sufficiently rapid, however, the rate of impurity rejection occurring at the rapidly growing interface can overcome the rate of diffusion of impurity away from the interface and cause the impurity concentration at the interface to be appreciably greater than that of the bulk of the melt. This enrichment lowers the melting point and can cause the rate of rapidly growing surfaces to decrease markedly.

The rate of crystal growth is also sensitively dependent on the temperature at the interface. This temperature and the temperature existing in the melt can differ significantly if the heat of fusion which is liberated upon crystallization is not quickly dissipated. The rate of heat transfer from the sample to the bath, dQ/dt, can be estimated for simple geometric forms of sample vessel immersed in a constant temperature bath. It can be shown that the rate is greatly increased by subjecting the sample to stirring.

Even in the event that the rate of mass and heat transfer is infinitely rapid, the rate of crystallization will be limited by the finite rate of formation of the crystalline structure. According to the literature there are four known mechanisms of crystal growth (1–4): upon a rough surface, by a screw dislocation, by two-dimensional nucleation upon a smooth surface, and by three-dimensional nucleation. The rate laws for these mechanisms are

$$\text{rough surface growth rate} = K_1\Delta T + A_1 \quad \text{(Eq. 1)}$$

$$\text{screw dislocation growth rate} = K_4\Delta T^2 \quad \text{(Eq. 2)}$$

$$\text{2-D nucleation growth rate} = K_2 \exp\left[-A_2/\Delta T\right] \quad \text{(Eq. 3)}$$

$$\text{3-D nucleation growth rate} = K_3 \exp\left[-A_3/\Delta T^2\right] \quad \text{(Eq. 4)}$$

where ΔT is the temperature potential and K_x and A_x are characteristic constants of the particular system studied. The rate laws derived in the literature, however, have been derived for ideally pure materials, and for this reason the temperature potential can be represented by the temperature difference between the freezing point and the water bath. This assumption tacitly assumes that the equilibrium temperature of the sample is remaining constant as freezing proceeds and is valid only for pure systems.

It would be most difficult to account for the effect of impurity upon the system due to adsorption and occlusion, but the effect of impurity upon the potential can be obtained by considering the thermodynamic equation used in equilibrium dilatometry (5), $N_2 = A_0\Delta T_f[f/(1-f)]$, where N_2 is equal to the mole fraction of impurity, A_0 is the cryoscopic constant, f is the fraction of the sample that is melted, and ΔT_f is equal to the difference between the freezing point of the mixture, T_m, and the equilibrium temperature of the sample, T_s, at the particular fraction melted, f. The fraction melted can be obtained from a dilatometric curve (5) and is equal to the melting dilation, H, divided by the dilation coefficient, D, of the sample. The dilation coefficient, D, is equal to the total change in height of the mercury in the constant-bore tubing of the dilatometer recorded for complete melting of the sample from the solid state. The melting dilation, H, on the other hand, is equal to the change in height recorded for only a partial melting of the sample from the solid state. The equilibrium temperature of the sample is easily obtained from these relationships, i.e., $T_s = T_m - [N_2/A_0][(1-f)/f]$. The temperature potential of the system, then, is given by the difference between the equilibrium temperature of the sample and the temperature of the water bath, T_b; therefore

$$\Delta T = T_s - T_b = T_m - T_b - \left[\frac{N_2(1-f)}{A_0 f}\right] \quad \text{(Eq. 5)}$$

This equation shows that the temperature potential of an impure system is equal to the temperature potential if it were pure plus a correction factor to account for the effect of the impurity. If Eq. 5 is substituted for the temperature potential, the resulting rate equation can be applied to impure systems

$$\text{rough surface growth rate} = K_1\left(C - \frac{E}{f}\right) + A_1 \quad \text{(Eq. 6)}$$

$$\text{screw dislocation growth rate} = K_4\left(C - \frac{E}{f}\right)^2 \quad \text{(Eq. 7)}$$

smooth surface growth rate =

$$K_2 \exp\left[\frac{A_2}{C}\left(\frac{f}{f - E/C}\right)\right] \quad \text{(Eq. 8)}$$

3-D nucleation growth rate =

$$K_3 \exp\left[\frac{A_3}{C^2}\left(\frac{f}{f - E/C}\right)^2\right] \quad \text{(Eq. 9)}$$

In the above: $C = (T_m - T_b + N_2/A_0)$ and $E = N_2/A_0$.

The above equations predict that the mechanism can be identified by obtaining rate *vs.* height data at a constant water bath temperature and plotting rate *vs.* $1/f$, $(\text{rate})^{1/2}$ *vs.* $1/f$, log (rate) *vs.* $f/(f - E/C)$, and log (rate) *vs.* $(f/[f - E/C])^2$, as only one plot should yield a straight line.

EXPERIMENTAL

Apparatus.—The apparatus essentially consisted of a water bath controlled to ±0.001° and a dilatometer. The latter apparatus was similar to that previously described (5) with the exception that it was constructed in one piece.

Procedure.—The dilatometer was prepared in the following manner. The sample and stirrer were loaded into a test tube and the open end was sealed to constant-bore tubing as previously described. The end of the dilatometer thus formed was connected by means of a T-tube to an ampul of mercury and to a vacuum pump. After evacuating the system, mercury was introduced by lifting the ampul of mercury until the desired amount of mercury was introduced into the dilatometer. The T-tube was removed and the dilatometer placed into the water bath at a temperature within the melting point range of the mixture to temper the sample overnight. The temperature of the water bath was then slowly dropped to room temperature. The dilatometer was then placed into a high pressure bomb and subjected to a pressure of 3,000 lbs./in.^{2}. This step was necessary in order to eliminate void space which otherwise obscures the data. Finally the dilatometer was placed into the water bath. To investigate transition rates the temperature of the water bath was changed sharply and the mercury height read with a micrometer microscope at intervals of 1 to 5 minutes. From an equilibrium melting curve the readings were converted to fractions of the sample melted, f. The rates as a function of the fraction melted were obtained from the slopes of the fraction melted *vs.* time plots of this data. In another method the temperature of the water bath was varied by increments. Fraction melted-time readings were recorded at each temperature setting for short intervals in order to limit the change in the fraction of the sample melted. By alternately applying a freezing and melting potential, the variation of the fraction melted was held to a minimum. In this way the fraction of the sample melted was approximately constant, and the slopes of the various fraction melted-time plots at each temperature setting yielded the rate at the particular temperature of the water bath This method allowed the simultaneous study of melting and freezing as a function of the temperature.

The first method, which maintained a constant water bath temperature and a varying fraction melted, was preferred as the water bath temperature was rigidly controlled and the fraction melted can be measured very accurately. The second method, on the other hand, requires a constant fraction melted and a varying temperature; yet the fraction melted must be allowed to vary in order to obtain a rate; in addition, heat transfer and mercury expansion can affect the results if the fraction melted-time readings are taken for too short a time. This method, however, enjoys the advantage of requiring much less time to complete a rate study.

Sources of Materials.—Three lots of methyl stearate were used in this study. Eastman Kodak reagent grade methyl stearate was recrystallized several times from methanol. This methyl stearate is obtained by fractional distillation of natural products, but a gas chromatogram indicated that it was contaminated by methyl palmitate. The melting point of this sample was 38.33° and it was estimated from a dilatometric melting curve that it contained 0.065 mole fraction of impurity. Methyl stearate was also obtained by the reduction of methyl oleate. A gas chromatogram showed that the sample obtained in this manner was relatively free from methyl palmitate. The melting point of this sample was 38.60° and it was estimated that it contained 0.042 mole fraction of impurity. Recrystallization from methanol three times and then from Skelley C three times increased the melting point to 39.05° and decreased the impurity to 0.004 mole fraction.

RESULTS AND DISCUSSION

Establishment of Mechanism of Crystal Growth.—Dilatometric investigations on the rate of crystal growth as a function of the degree of supercooling were carried out on the three relatively pure lots of methyl stearate. Essentially the method employed was based on continual determination of the fraction melted of a partly solidified and slowly freezing sample of the lipid. The measurements are reported in terms of the fraction of the sample melted obtained by measuring the heights of the mercury column as observed in the dilatometer, the total height change for complete freezing from the liquid state being of the order of 40 cm. The sample was brought to near equilibrium at such temperature 38.75° that approximately 75% was in the liquid state. The temperature was then dropped sharply by a few tenths of a degree and the capillary height of the dilatometer was observed from time to time. A plot of the fraction melted *vs.* time was prepared and the rates at various degrees of crystallization were recorded.

In Figs. 1 and 2 these rates of crystal growth have been plotted as several functions of f, the melting dilation expressed as fractions of the sample melted. It is evident that according to preceding discussions, the rates appear to obey Eq. 8 or 9, based on the two- or three-dimensional nucleation as the rate determining step.

Since the above run was determined on a system largely melted, a run at a lower degree of melting was carried out to differentiate between these two

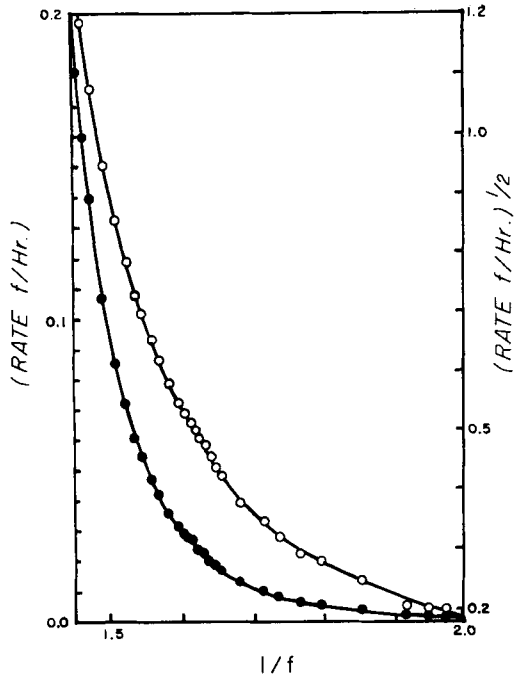


Fig. 1.—The rate data of the same experiment plotted according to Eqs. 6 and 7. ●, rate *vs.* $1/f$. ○, $(\text{rate})^{1/2}$ *vs.* $1/f$.

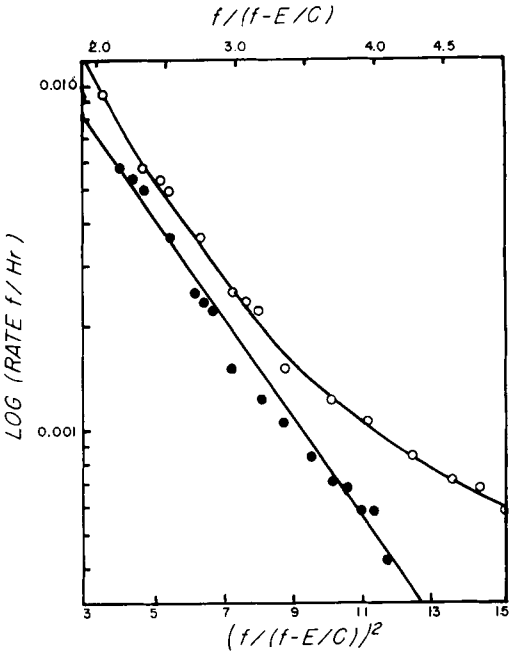


Fig. 3.—The rate data of the same experiment plotted according to Eqs. 8 and 9. This experiment was conducted at a relatively low degree of sample melting. ●, log(rate) *vs.* $[f/(f - E/C)]$. ○, log(rate) *vs.* $[f/(f - E/C)]^2$.

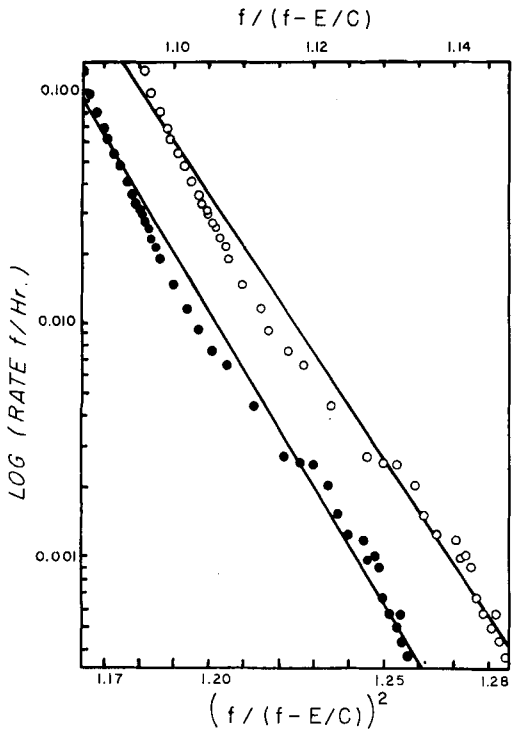


Fig. 2.—The rate data of the same experiment as Fig. 1 plotted according to Eqs. 8 and 9. ●, log(rate) *vs.* $[f/(f - E/C)]$. ○, log(rate) *vs.* $[f/(f - E/C)]^2$.

mechanisms. The results are shown by Fig. 3 and indicate that the crystal growth is governed largely by the intrinsic rate of formation of crystal planes.

To substantiate this the rate of phase transformation under fixed conditions except for temperature was determined as a function of temperature. In this method the equilibrium temperature of the sample at its observed height was first obtained from its dilatometric melting curve (5). A freezing potential was then applied to the system by sharply dropping the water bath temperature, the fraction melted-time readings being taken, however, only for a sufficient time to establish the rate. The sample then was remelted until the fraction of the sample melted had increased to its initial point and then the temperature of the water bath was again sharply dropped to another temperature. In this manner the rates of crystallization were obtained directly as a function of the supercooling, $T_a - T_b$. Figure 4 shows that a plot thus determined of the log (rate) *vs.* $1/\Delta T$ is a straight line verifying the previous results that the rate law which governs this system is that the rate $= K_2 \exp(-A_2/\Delta T)$. This strongly suggests that the rate determining process for crystal growth in this system was nucleation of crystal planes, the extension of the nucleus to the edges being relatively rapid. Figures 1 to 4 were determined using the sample containing 0.004 mole fraction of impurity. All samples, however, displayed the same behavior.

Melting Characteristics of Methyl Stearate Crystals Formed under a Moderate and Extremely Small Temperature Gradient.—The melting behavior of methyl stearate was found to be a rather sensitive function of its immediate history in a

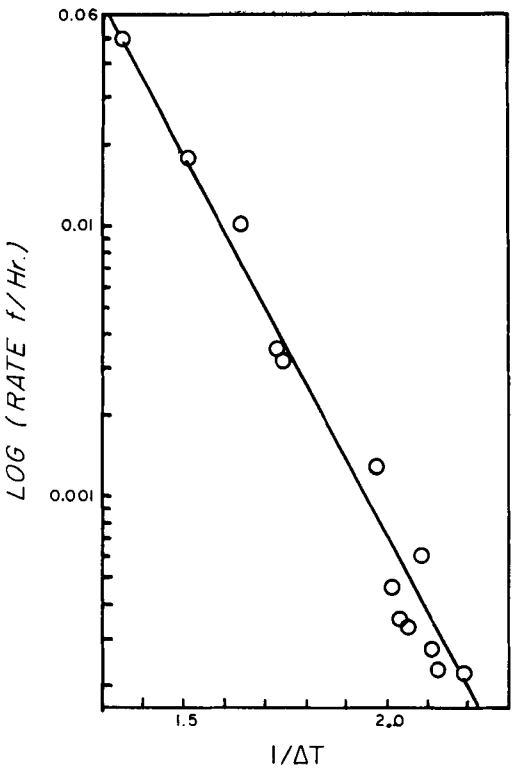


Fig. 4.—The log(rate) as a function of $1/\Delta T$.

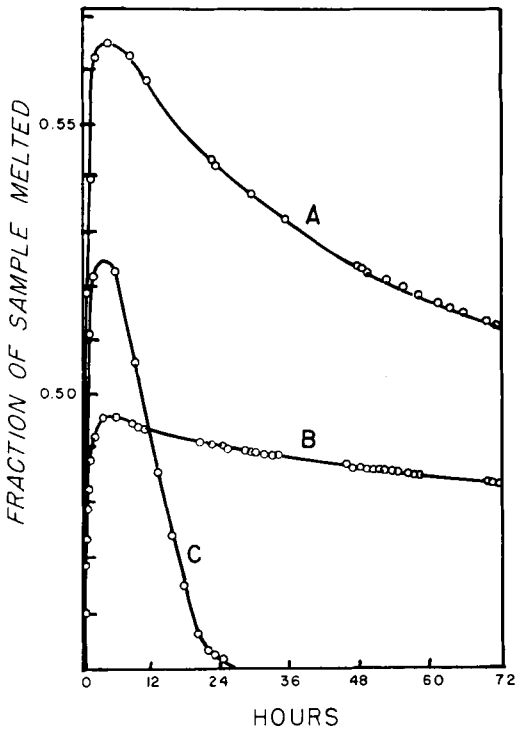


Fig. 5.—The effect of past history upon "after-melting" contraction. Curve A, initial run where $T°$ was changed from 37.08 to 37.50°. Curve B, rerun after freezing the sample very slowly with the aid of stirring where $T°$ was changed from 37.08 to 37.50°. Curve C, third run after freezing the sample very rapidly without any stirring where $T°$ was changed from 36.76 to 37.29°.

manner not associated with polymorphic transitions. When the system was exposed to a melting potential, the sample was observed to contract after initially expanding for a period of time. Although the rate of contraction decreased with time, the recovery process persisted as long as 7 days without stopping. This peculiar behavior will be referred to as "after-melting" contraction, and is illustrated by curve A of Fig. 5 which shows the fraction of the sample melted readings recorded after applying a melting potential to the system.

This phenomenon can be possibly rationalized as being due to imperfections in the crystal structure induced by excessive growth rates. The crystal eliminates these stresses by overmelting and then refreezing. Impurity can exert the same effect if its distribution is not thermodynamically stable within the solid sample as it will redistribute itself during melting.

If either of the above is responsible for "after-melting" contraction, then, crystallizing the sample under equilibrium conditions should tend to diminish its effect. The sample was melted and then solidified using a small temperature potential and with the aid of stirring. The results of the rerun are shown by curve B in Fig. 5. Comparison with A shows that "after-melting" contraction was significantly eliminated. To check this further the sample was again melted and solidified using a large temperature potential without the aid of stirring. Curve C in Fig. 5 shows that the effect of these harsh conditions greatly increased "after-melting" contraction and seems to indicate that the above explanations may be true. Although samples of varying purity were used, the effect always appeared. This seems to indicate that "after-melting" contraction may be largely due to the presence of crystal imperfections.

Influence of a Thermal Gradient on the Rate of Melting.—The mechanism of melting was investigated, and it was found that a plot of the melting rate *vs.* the reciprocal fraction melted yielded a straight line. Some of the runs, however, revealed a break in the curve between the rates of 3,000 to 5,000 mg./min. This suggests that the determining factor at high melting rates differs from that which is operative at the lower rates. This is illustrated by Fig. 6.

According to Eqs. 1 and 6, a plot of the rate *vs.* the reciprocal fraction melted will be straight in the event the rate is first order with respect to the temperature potential. In this case, either melting from a rough surface is occurring or heat transfer is the governing factor. For this study the rate of heat transfer from the bath to the sample, dQ/dt, in the presence of stirring can be calculated from

$$\frac{dQ}{dt} = \frac{K_g A \Delta T}{n} \text{ cal./sec.} \qquad \text{(Eq. 10)}$$

where n is the thickness of glass in dilatometer, K_g is the heat conductivity of glass, A is the area of the glass, and ΔT is the temperature potential. The theoretical rate of heat transfer calculated from

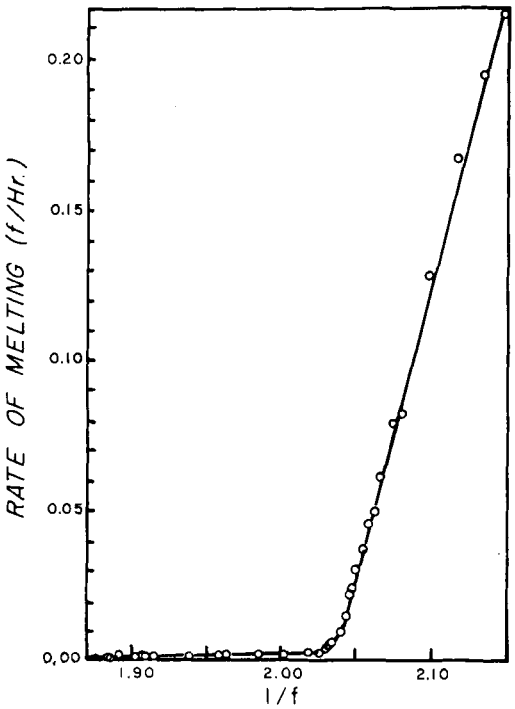


Fig. 6.—The rate of melting as a function of the reciprocal fraction melted.

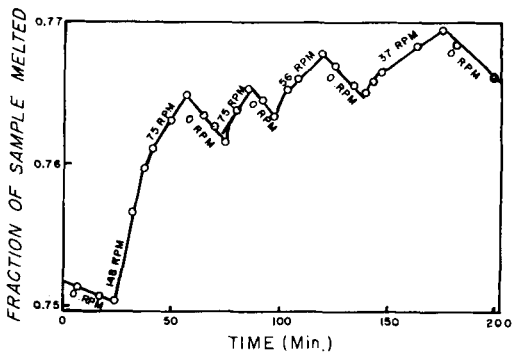


Fig. 7.—The effect of stirring upon the fraction of the sample melted *vs.* time curve.

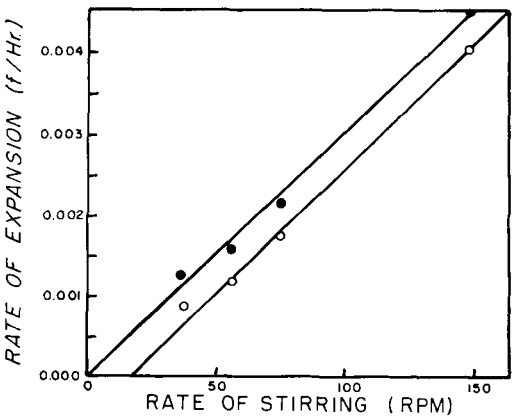


Fig. 8.—The rate of expansion as a function of the rate of stirring. O, the observed rate; ●, the net rate (the observed rates with stirring less the observed rate of the unstirred sample).

the above is approximately 100 cal./°C./min. If the governing rate is the rate of heat transfer, then Eq. 10 predicts that the value of K_1 in Eq. 1 will be equal to $(K_0A)/n$ or 100 cal./°C./min. At the same time, Eq. 6 predicts that the value of K_1 can be calculated from the slope of the rate *vs.* reciprocal fraction melted curve as the slope is equal to $(K_1N_2)/A_0$. The values of K_1 which were calculated from the beginning slopes of a few runs seem to indicate that heat transfer is the governing factor at fast rates of melting as the values of K_1 were approximately 100 cal./min.

The slope of the straight line after the break varied greatly, indicating that the slope may depend upon experimental conditions. A possible cause may be the effect of "after-melting" contraction, which varies in degree from one run to another depending on the past history. It may also be possible that the rate is not proportional to ΔT in this region, but may be proportional to some other function of ΔT.

Since the first part of the curve was not determined by the rate of melting, only the slower rates can be used to determine the rate law; at low potentials, however, the rates become very sensitive to the effects of heat transfer, "after-melting" contraction, temperature, surface area, and other experimental conditions. Since these variables complicate the rate data, the mechanism of melting was not investigated any further.

Apparent Influence of Stirring on the Crystallization Rate.—An unstirred sample which was solidifying at a slow rate was observed to undergo the reverse process when stirring was initiated and refreeze when stirring was stopped. A system of pulleys which allowed the rate of stirring to be varied over a wide range was utilized to study this peculiar behavior further. A typical plot of the height *vs.* time with a varying rate of stirring is shown by Fig. 7. The slopes of the curve at the various rates of stirring were obtained, and the rates were plotted *vs.* the rate of stirring expressed as revolutions per minute (r.p.m.). The plot of the observed and net rates are shown in Fig. 8, revealing straight line relationships. The exact reason for this behavior has not been established.

Discussion.—Previous studies involved measurements of the linear rate upon one plane of the crystal, maintained a constant surface area, utilized highly purified substances, and relied upon diffusion for all transport processes. This study, however, utilized samples whose purity varied over a wide range and involved dilatometric measurements of the combined growth of all planes of a variable number of crystals. Most important, however, the conditions of this study more closely resemble those of industry (free growth with stirring) which permit the results to be more readily applied to industrial systems.

The study of the rates of phase transitions can yield useful information which can be utilized to advantage in the production and storage processes

by the pharmaceutical industry. Many of the substances used in the formulation of medicinals are polymorphic and frequently exhibit melting points, solubilities, pharmacological activities, and other important properties which differ markedly from each other; as a result, the conversion of one form to another upon aging presents an important problem, as it can often curtail the shelf life of products.

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Notes

Chemical Effect of High Level Gamma Irradiation on Blood Glucose *in Vitro*

By LINDA KAY RAMES† and HAROLD S. BAILEY

Furan-2,5-dicarboxylic acid is one of the decomposition products of blood glucose subjected to high energy gamma irradiation.

THE EFFECT of ionizing radiation on materials of biological interest is important to our understanding of radiation hygiene. Pioneering studies have been made on the changes produced in amino acids, proteins, carbohydrates, and other biochemicals upon bombardment with nuclear radiation.

Changes in aqueous solutions of biochemicals upon irradiation are complex. Due to free radical formation, a large quantity of hydrogen is formed upon irradiation of water containing traces of halides. Also, dissolved oxygen in irradiated water will react with (H) radicals present to form the free radical (HO_2). Therefore, oxidizable or reducible solutes present in an irradiated aqueous medium may be readily oxidized or reduced.

Because of its importance to living organisms, we have been interested in the effect of ionizing radiation on blood glucose.

It is possible that the effect of irradiation of blood glucose and other blood constituents may provide an insight into the mechanism of radiation sickness. Since blood acts as a transportation agent for the body and as a solvent for so many complex compounds, it could be the source or carrier of toxic decomposition products resulting from irradiation.

Radiation effects on blood glucose have been noted both *in vitro* and *in vivo* (1–4). Irradiation of both dogs and rats has resulted in the development of hypoglycemia.

One could expect that the effects of ionizing radiation on blood might produce several by-products from blood glucose since blood would yield many other ions and radicals besides those of water upon irradiation. These other substances may alter the process of decomposition or react quickly with the products.

Previous research by us indicated that furan-2,5-dicarboxylic acid might be one of the irradiation decomposition products of glucose solutions. The objective of this study, therefore, was to show whether furan-2,5-dicarboxylic acid is produced upon irradiation of blood.

EXPERIMENTAL

Irradiation of Blood Plasma.—Rabbit blood plasma was chosen as the medium in which the effects of gamma radiation on blood glucose would be studied. Heparinized blood was centrifuged, the plasma decanted, and kept under refrigeration. Some hemolysis occurred.

A total of 9.01 mg. of radioactive glucose randomly-labeled with carbon^{14} was added to 8 ml. of the blood plasma contained in a small beaker. The specific activity of the radioglucose was 1.0 mc. per mmole, and a total activity of 50 μc. was present. After solution was evident, the blood plasma was poured into a half-ounce prescription bottle, and the beaker was rinsed twice with approximately 4-ml. portions of blood plasma, the rinsing combined with the original solution in the bottle.

For irradiation, the bottle containing the labeled blood plasma was fastened to a pegboard constructed in such a manner that the cobalt^{60} irradiation source could be positioned at a known distance by remote control. The source was placed exactly 3 inches from the bottle.

The Co^{60} source had a gamma ray activity of 103.6 curies. At a distance of 3 inches the blood plasma received an approximate dose of 23,760 roentgens per hour or a total of approximately 1,200,000 roentgens in the 50.5 hours of irradiation.

Radiochemical Analysis by the Carrier Technique.—A 100-mg. portion of synthesized furan-2,5-dicarboxylic acid was added to the irradiated plasma. The carrier furan-2,5-dicarboxylic acid was then solated from the irradiated system by the following process.

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Accepted for publication July 19, 1961.
Abstracted in part from a dissertation presented to the Graduate Division of South Dakota State College by Linda Kay Rames in partial fulfillment of the requirements for the Master of Science degree, August 1960.
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The irradiated blood plasma was heated until a brownish-gray, dry mass was produced and, after cooling, 100 ml. of 50% sulfuric acid was added to the residue. After heating at 180° for 1 hour, the resulting mixture was cooled and diluted with distilled water, filtered, and the carbonaceous material on the filter washed with two portions of a 1:1 ethanol-water solution.

The filtrate was heated to remove some of the ethanol in which the furan-2,5-dicarboxylic acid is very soluble. When the volume was reduced to 100 ml., heating was stopped and the solution cooled. The acid filtrate was then successively extracted in a continuous liquid-liquid extractor with 75-ml. portions of ether. A total of 525 ml. was used for the extraction. After evaporation of the ether extract, the residue was dissolved in 50 ml. of hot alcohol, filtered, and evaporated down to dryness in a tared counting planchet.

A radioassay of the residue in the planchet was performed using a windowless gas counter. The weight of the residue assayed was 58.7 mg. and the activity was 89,456 c.p.m., corresponding to a specific activity of 1,524 c.p.m. per mg.

The planchet was then placed in a beaker and the residue dissolved in hot alcohol, activated charcoal was added and the material boiled, filtered hot, evaporated, and radioassayed in the same manner as the first sample.

The white crystalline residue weighed 11 mg. with an activity of 14,424 c.p.m., corresponding to a specific activity of 1,311 c.p.m. per mg.

The residue was purified a third time with a final sample weight of 10.7 mg. with an activity of 14,775 c.p.m. corresponding to a specific activity of 1,381 c.p.m. per mg.

A distilled water solution of the final residue showed ultraviolet absorption characteristics similar to that of irradiated glucose solutions and synthesized furan-2,5-dicarboxylic acid.

DISCUSSION

Though furan-2,5-dicarboxylic acid was isolated as a metabolic product by Flashentrager, *et al.* (5), as early as 1937, its physiological action and biochemical transformation have not been extensively studied.

Research (6, 7) has indicated that the formation of furan-2,5-dicarboxylic acid in a biological system is dependent on the presence of glucuronic acid or galacturonic acid rather than that of glucose. This indicates that the first step in the metabolic formation of furan-2,5-dicarboxylic acid from glucose would require oxidation to glucuronic acid or rearrangement and oxidation to galacturonic acid.

The formation of radicals from the ionization of water provides a medium in which these oxidative processes are probable.

The formation of radicals during the irradiation of blood would be enhanced by the presence of halide ions. Of course, the complexity of blood would lead to the formation of ions and other radicals besides (H) and (OH).

Evidence indicates that the second step in the formation of furan-2,5-dicarboxylic acid would be formation of ketohexose by further oxidation or rearrangement of glucuronic acid (7).

Ring closure of the ketohexose would yield a heterocyclic ring containing oxygen with substituents on carbons 2 and 5. Dehydration of this structure by removal of two hydrogen and two hydroxyl groups would yield the furan ring. Evidence has already been produced proving that oxidation of the substituents on carbons 2 and 5 of the furan ring occurs readily (7).

Under the conditions of our experiment it is apparent that furan-2,5-dicarboxylic acid is a decomposition product of blood glucose upon irradiation of blood plasma *in vitro*.

The presence of radioactivity in the sample of furan-2,5-dicarboxylic acid indicates beyond a doubt that this product resulted from a transformation of the radioglucose added to the blood plasma.

The difference in activity of the purified and unpurified samples was probably due to large quantities of degradative fragments of radioglucose and possibly other organic substances formed by the action of the concentrated sulfuric acid. Since other radioactive compounds can be formed, it is essential that the furan-2,5-dicarboxylic acid isolated be purified to a constant specific activity in order to state that the activity is due mainly to radioactive furan-2,5-dicarboxylic acid. It can be seen from a comparison of the specific activities of the second and third isolations (1,311 c.p.m./mg. *vs.* 1,381 c.p.m./mg.) that purification of the furan-2,5-dicarboxylic acid was adequate for our conclusions. In addition, the ultraviolet absorption characteristics of the isolated product agreed with those of synthesized furan-2,5-dicarboxylic acid.

CONCLUSIONS

1. A study of a chemical effect of high level gamma irradiation on blood glucose *in vitro* using the radioactive tracer technique has been made.
2. Furan-2,5-dicarboxylic acid was shown to be one of the decomposition products formed from high level gamma irradiation of blood glucose *in vitro*.
3. A further study of the effect of irradiation of blood on blood glucose with radioactive glucose would be feasible to prove the identity of other possible decomposition products.

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Effects of the Potassium and Magnesium Salts of Aspartic Acid on Metabolic Exhaustion

By H. ROSEN, A. BLUMENTHAL, and H. P. K. AGERSBORG

Two groups of 40 rats were placed in a swim test situation designed to produce metabolic exhaustion. The provision of potassium and magnesium aspartates to one group at a dose of 1 Gm./Kg. significantly delayed the onset of metabolic exhaustion as evaluated by swimming time. It is considered that this alteration in swim time occurred as a result of the material's affecting those animals which would ordinarily have swum for less than 196 minutes rather than altering the times of the group as a whole.

THE SCIENTIFIC literature is replete with reports on various rat swim test situations. Included are the effects of age and sex differances (1), fatigue, practice and drug effects (2), the shortening of swim time by the application of weights (3, 4), and the ability that diet has to alter performance (5, 6).

Some of the most impressive data relating to swim tests have been reported by Richter (7). In these experiments it was demonstrated that a direct relationship exists between duration of swim time and the temperature of the water in which the animals are swimming. Additionally, a presentation of a possible causal relationship between those animals swimming "indefinitely" and for "very short" periods was made.

None of the publications reviewed made a point of evaluating metabolic exhaustion. It is assumed that an animal, faced with a swim or drown situation, will perform to his utmost ability. Although this assumption must be true, there are no data to indicate whether this level of ability is dictated by emotional factors (7), availability of metabolic substrate, or accumulation of toxic end products.

EXPERIMENTAL

To determine these points and to determine the size and type of container, temperature, optimum number of animals per volume of container and, most important, to establish sufficient knowledge leading to a recognizable and reproducible end point, numerous orientation studies were made prior to final experimentation.

The "swimming pool" finally adopted was a rectangular, custom-made aquarium 36 in. long, 24 in. wide, and 24 in. deep; the tank was equipped with a thermoregulator and heater to maintain constant temperature and a water circulator to facilitate maintenance of constant temperature without either aiding or antagonizing the normal swimming efforts of the rats. The water depth in all reported tests was 20 inches.

In preliminary tests, male rats ranging in weight from 150–300 Gm. were used. It was concluded that any male rats within this range would be satisfactory. However, for the reported experiments, this range was narrowed to 180 to 245 Gm.

When rats are placed in water they first swim in a horizontal position on the surface; then, looking for a means of escape, they often go as deep as the bottom of the tank; next they assume a vertical position at the surface and use all four legs vigorously, with occasional second-long rest periods. In short order, they must work continuously to keep their heads above water. At this stage, while the hind legs pump steadily, the animals fold their forelegs in front of the chest in an apparent effort to use them as wings or fins to avoid the sinking process. As work continues, they frequently sink and must struggle to reach the surface. Eventually loss of control of movements is observed and the animals sink to a depth of 10 to 20 in. with an inability to fight to the surface; some will walk the perimeter of of the tank bottom, some will remain almost motionless at a depth of 10 or more inches, while others flail aimlessly without rising. These are variations of the end point. Rats removed from the water at this time usually recover. A rat in this condition is finished swimming as far as physical ability is concerned; he is fatigued and apparently near exhaustion. This is the first reproducible and constant symptom which is always observed; hence it was selected as the end point.

If rats are allowed to remain in the water when the end point is reached, a few recover sufficiently in about 30 seconds to make a few more trips to the surface. Those that show no recovery remain under water and drown. These latter observations are not routine; the rats were removed from the tank at the end point, but early in the series some were allowed to remain in order to provide terminal observations.

It was found that a census of 8 to 10 rats was optimum for the tank used; thus, when the number of rats was reduced below eight, additional rats were added to maintain that number.

Preliminary observations concerning onset of metabolic exhaustion were made by exposing the animals to various ambient water temperatures, observing the swim time, resting for either 15 or 30 minutes, and exposing the same animals to the same conditions of temperature and swim. At the lower temperatures, 20°, the animals were capable of approximately 20 minutes of swim; this could be reproduced for several exposures. As the water temperature was increased, it was found that swim time increased in accordance with Richter, but that duration of time of subsequent exposures was appreciably reduced. This was taken to mean that metabolic involvement in the initial swim was such that complete recovery had not taken place by the time the second effort was required.

Since normal temperature regulating mechanisms are considered to be in balance at approximately 85° F. (29° C. environmental), and, since metabolic activity would be neither inhibited nor enhanced by

Received October 10, 1961, from the Research Division, Wyeth Laboratories, Inc., Philadelphia 1, Pa.
Accepted for publication October 18, 1961.

exposure to such an ambient temperature, the reported tests were done at a water temperature of 28.0 ± 0.25° C. Control animals, after swimming to exhaustion and given 15-minutes rest, were usually unable to swim at all when exposed for the second time to the water temperature. It was concluded, therefore, that a situation producing metabolic exhaustion had been produced.

Laborit (8, 9) has demonstrated that several agents have the ability to prolong the duration of rat swim time. The most active substance reported was a combination of the potassium and magnesium salts of aspartic acid. Since control rats, in the Laborit technique, were able to swim for appreciably shorter times than those in this study, and, since second exposure resulted in an observed time approximating that of the first, there was felt to be a need for additional data more directly related to metabolic exhaustion as described.

For the actual test, 10 male rats apparently healthy and ranging in weight between 180 and 245 Gm. were selected on the afternoon preceding the test day. They were arranged into two groups of five each, weighed, and small slits made in the ears for purposes of identification. One group was treated twice by stomach tube with 500 mg./20 ml./Kg. each of potassium aspartate and magnesium aspartate (1000 mg./20 ml./Kg. total) in one solution. The first dose was given 17 hours before the test and the second 45 to 60 minutes before the test. The control rats were handled in a like manner using water only; both groups were allowed food and water *ad libitum* until actually placed in the tank for swimming.

At zero time, both groups of rats were placed in the pool under the conditions previously described and allowed to swim until each, in turn, reached the end point of fatigue. As each rat reached his end point he was removed from the water, identified, and his total elapsed swim time recorded.

It should be remembered that only swim time is assayable by this test; no attempt at measurement of work output is possible.

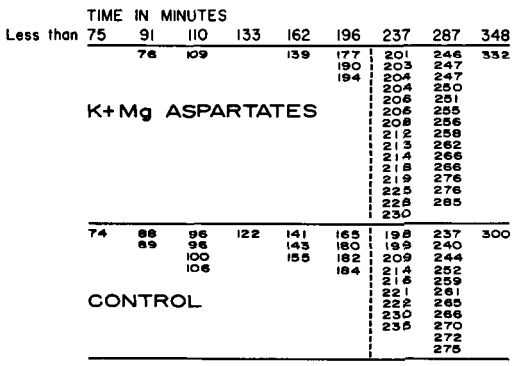


Fig. 1.—Average of the control times and aspartate animals times.

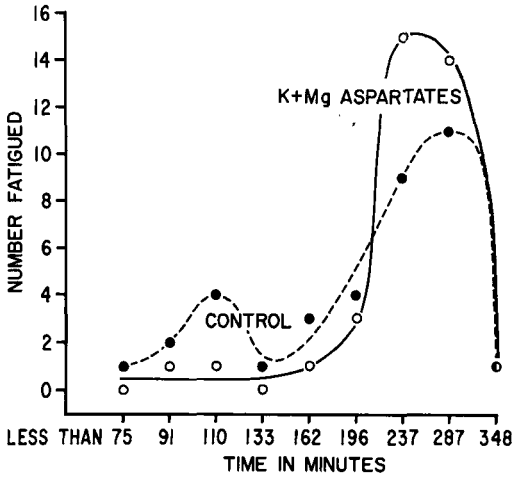


Fig. 2.—Plots of data in Fig. 1.

RESULTS AND DISCUSSION

The accompanying figures present the results obtained from eight cumulative experiments; they offer similar data, in a different way. Thirty-six control and 36 aspartate treated rats are included; the remaining eight will be discussed later. The rats are divided into log-spaced swim time groups.

An average of the control times and aspartate animals' times in Fig. 1 indicates that the control rats swam 194.6 ± S.E. 7.96 min. and that the drug-treated rats swam 223.6 ± S.E. 8.05 min., an increase of 15% in swimming time.

If the data presented in Fig. 1 are plotted graphically (Fig. 2), the distribution of the controls is seen to be bimodal. In both groups there appears to be a line of demarcation at 196 minutes; 41.7% of the controls swim less and 58.3% swim more than 196 minutes. On the other hand, the aspartate-treated rats shift to a unimodal frequency; 16.7% swim less and 83.3% swim more than 196 minutes. Application of the chi square test indicated that this shift was significant at a level less than 5%. It is felt that this is a strong indication that the swimming times of rats which have longer endurance are not altered by the aspartates while the swimming time of animals of lesser endurance are prolonged.

The eight rats which are not represented in the figures but which are included in the calculations were discarded for reasons as follows: one aspartate rat was obviously incapable of coordinated swimming; he was removed from the water in about 20 minutes and expired shortly thereafter. Four control rats and three aspartate rats were interminable swimmers; they were swimming well after 6 hours and were discarded. As a check on our interpretation, one of the control rats and one aspartate rat were timed further; they were still swimming after 8 hours and were removed from the tank.

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Determination of Nikethamide with Cyanogen Bromide and Barbituric Acid

By O. PELLETIER and J. A. CAMPBELL

Two procedures are presented for the determination of nikethamide which involve its reaction with cyanogen bromide and barbituric acid. The first method is highly specific while the second is more rapid but slightly less specific. These methods have the advantage of being simpler, more rapid, and more specific than the present N.F. procedure.

WHILE testing various pyridine derivatives in the cyanogen bromide-barbituric acid method recently developed in this laboratory for the determination of niacinamide (1), it became evident that under certain conditions the diethylamide of nicotinic acid (nikethamide) also produced a pink color with these reagents which might be useful for its estimation.

The present N.F. method (2) involves a titration of the diethylamine after digestion and distillation without differentiating other nitrogen-containing compounds. Since this procedure was not specific it was decided to investigate the color reaction. This paper describes two possible methods for determining nikethamide and the studies that were made in developing these methods.

METHOD

Reagents.—*Cyanogen Bromide,* 10%.—Prepare and use under hood. Do not let cyanogen bromide or solution come in contact with skin. Dissolve a weighed amount of cyanogen bromide in warm water at about 40°. Allow to come to room temperature and dilute to 10% solution. Store in refrigerator and bring to room temperature before using.

Potassium Phosphate, Monobasic, 3%.—Dissolve 15 Gm. KH_2PO_4 in water to make 500 ml.

Potassium Phosphate, Monobasic, 0.3%.—Dilute 100 ml. of 3% KH_2PO_4 to 1000 ml. with water.

Barbituric Acid Buffered Solution, Saturated (2%).—Prepare volume required for each batch of assays by adding 100 ml. of 3% KH_2PO_4 to each 2 Gm. of reagent grade barbituric acid and shaking vigorously at intervals for about 1 hour. Filter before using.

Assay.—*Nikethamide Standard Solution.*—Dilute an aliquot of nikethamide N.F. 25% standard[1] with 0.3% KH_2PO_4 to 3.0 mcg. per ml.

Assay Solution.—Dilute to 3 mcg. per ml. with 0.3% KH_2PO_4.

Reaction.—Run in duplicate. To 1.0 ml. of standard or assay solution in colorimeter tube, add 0.5 ml. cyanogen bromide, mix, stopper, and let stand 10 minutes. Add 10 ml. barbituric acid, mix, and let stand 5 minutes. Place tubes in water bath at 50° for 25 minutes and then transfer to another bath at 20° for 5 minutes. Within the next 15 minutes set spectrophotometer at 0 absorbance at 560 mμ with a standard blank in which cyanogen bromide is replaced by water and measure absorbance of reaction product.

Calculations.—Mcg. per aliquot = $Au \times 3/As$, where Au = absorbance of unknown and As = absorbance of standard.

RAPID METHOD

Dilute standard with 0.3% KH_2PO_4 to 1.0, 1.5, 2.0, 2.5, and 3.0 mcg. per ml., respectively, and dilute sample to about 2 mcg. per ml. To 1.0-ml. standard solution or assay solution (in duplicate) in colorimeter tube, add 0.5 ml. cyanogen bromide, mix, stopper, and let stand 10 minutes. Add 10 ml. of barbituric acid, mix by swirling, and read maximum absorbance which remains stable from 10 to 15 minutes after addition of barbituric acid. Set spectrophotometer at 0 absorbance at 505 mμ with a standard blank in which cyanogen bromide is replaced by water. Plot absorbance of standard *vs.* concenration and draw line of best fit but do not consider the origin as a point in drawing the line. Read from curve the number of mcg. per ml. corresponding to absorbance of sample.

EXPERIMENTAL RESULTS AND DISCUSSION

Factors Affecting the Reaction.—Modifications of the niacinamide method previously presented (1) were thoroughly investigated. The optimum time of cyanogen bromide reaction was 10 (±1) minutes. When the reaction was conducted at room temperature, maximum absorbance was obtained at 505 mμ 10 minutes after addition of barbituric acid. There was also a minor peak at 560 mμ which reached its maximum in about 2.5 hours. Development of the latter peak could be accelerated by incubating 25 minutes at 50° after allowing the barbituric acid reaction to proceed 5 minutes at room temperature. The use of other temperatures offered no advantages for this determination.

Concentration of Nikethamide.—At 560 mμ there was a linear relation between absorbance and concentrations up to 10.0 mcg. which passed through

TABLE I.—PERCENTAGE OF ABSORBANCE OF NIACIN DERIVATIVES IN COMPARISON TO NIKETHAMIDE (100%)

Substance	Regular Method,[a] %	Rapid Method,[b] %
Nikethamide	100	100
Niacin	none	0.5
Niacinamide	none	7.4
Pyridine-3-sulfonic acid	1.2	1.9
Nicotinic acid dimethylamide	none	9.1
Nicotinic acid methylamide	none	3.6
Nicotinic acid ethylamide	1.2	1.4
Nicotinic acid methyl ester	none	2.5
Nicotinic acid ethyl ester	none	2.5

[a] Substance tested at 3 mcg. [b] Substance tested at 2 mcg.

Received July 6, 1961, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

Accepted for publication September 13, 1961.

[1] Proposed standard, containing 25% nikethamide according to the N.F. requirements (2).

the origin. At 505 mμ, concentrations from 1.0 to 3.5 mcg. gave a straight line which did not pass through the origin. In this latter case a standard curve was used to calculate the results. The mean recoveries at different levels were 100.4 ± 2.7% for 7 determinations with the first method and 100.1 ± 2.5% for 6 determinations with the second method.

Reaction of Related Compounds.—To test the specificity of the methods, compounds most likely to react similarly to nikethamide were assayed at the 3-mcg. level for the regular method and at 2 mcg. for the rapid method. Results are reported in Table I in terms of percentage of absorbance of related compound in comparison to nikethamide. For the regular method, the highest percentage was 1.2% for pyridine-3-sulfonic acid and nicotinic acid ethylamide; other substances tested gave no absorbance. For the rapid method, the highest percentages were 9.1% for nicotinic acid dimethylamide and 7.4% for niacinamide; others were below 4%. The fact that nikethamide is practically the only compound giving a colored product seems to be due (*a*) to the different rate of reaction of various pyridine derivatives with cyanogen bromide and (*b*) to the difference in stability of the colored compound formed upon reaction of the pyridinium derivative with barbituric acid unsubstituted in the 5 position.

It may be concluded that the regular method is more specific than the rapid method although the latter would be quite satisfactory for most routine checking purposes.

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Citrinin from *Penicillium steckii* Zaleski

By A. JABBAR and A. RAHIM

The antibiotic substance isolated from cultured *Penicillium steckii* Zaleski was identified as citrinin.

PENICILLIUM STECKII Zaleski[1] was shown to have antibiotic activity against *Staph. aureus* and *Ps. pyocyanes* (1). It was later found that the same strain formed a penicillin-like antibiotic (2). In this paper the isolation, characterization, and crystallization of the antibiotic is described and it is confirmed that the antibiotic is citrinin.

The organism *Penicillium steckii* Zaleski was cultivated in Czapek-Dox solution containing 0.25% yeast extract; the active principle from the potent greenish culture broth was isolated by chloroform, and purified by adsorption chromatography on acid-washed alumina. The antibiotic was crystallized in prismatic needles and in plates from chloroform and petroleum ether mixture. The crystalline sample, melting at 165–169° (with decompn.), is readily soluble in chloroform and acetone. LD_{50} is 100 mg. The determination of its functional groups and the studies of its physicochemical properties show that the antibiotic is citrinin.

EXPERIMENTAL

Culture Media.—Czapek-Dox solution; sucrose, 20 Gm.; sodium nitrate 2.0 Gm.; potassium dihydrogen phosphate, 1 Gm.; potassium chloride, 0.5 Gm.; magnesium sulfate, heptahydrate, 0.5 Gm.; ferrous sulfate, heptahydrate, 0.01 Gm.; water to 1 L. The only source of nitrogen in the medium is sodium nitrate which corresponds to 0.3296 Gm. of nitrogen per L. Czapek-agar medium was the best for sporulation.

Production and Isolation of the Antibiotic on Czapek-Dox Solution.—For the production of the antibiotic, the surface culture was found to be better than the shake culture. Of the different carbon sources such as glucose, sucrose, lactose, and galactose, sucrose was the best. Grain extract, beef extract, and yeast extract were found to have a stimulatory effect both on the growth of the organism and on the production of the antibiotic.

Czapek-Dox solution containing 0.25% yeast extract was distributed in 50-ml. amounts in 250-ml. conical flasks which were plugged with cotton and sterilized at 15 lb. pressure for 20 minutes. The contents of the flask were then inoculated with spore-suspension in water, prepared from Czapek-agar slopes of *Penicillium steckii*, one flask being sown from each slope. The flasks were incubated at room temperature for 4 days. The culture fluids were separated from the mycelia by decantation. The filtrates were then extracted with an equal volume of chloroform. For the isolation of the antibiotic present in the mycelia, the thoroughly washed mycelia were crushed with sand and extracted with alcohol. The extracts were evaporated to dryness at 50° under reduced pressure, taken into chloroform, and chromatographed on acid-washed alumina to remove the coloring matter. The antibiotic was crystallized from chloroform and petroleum ether mixture. Repeated crystallization gave lemon-yellow prismatic needles, sometimes lemon-yellow plates, melting at 165–169° with decomposition.

The purified product is readily soluble in chloroform and acetone, very sparingly soluble in water. It is soluble in alcohol, aqueous alkali, less soluble in ether, still less in petroleum ether.

Received May 10, 1961, from the Department of Biochemistry and Pharmaceutical Chemistry, Dacca University, Dacca, East Pakistan.
Accepted for publication June 8, 1961.
The authors are grateful to Miss Brown of Imperial Mycological Institute, Kew, U. K., for her kind identification of the organism and to Prof. W. B. Whalley, Dept. of Organic Chemistry, University of Liverpool, for his kind supply of sample of citrinin.

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The antibiotic was found to be active *in vitro* against the following test organisms

Organisms	Activity[a]
Bacillus subtilis (CN663)	4+
Proteus vulgaris (CN2770)	+
Streptococcus aureus (CN2)	+
Staphylococcus aureus (CN4108)	2+
Pseudomonas aeruginosa (NCIB8295)	+
E. coli (CN311)	+
Vibrio cholerae	
Ogwa strain	3+
Inaba strain	2+
Paratyphoid B	+

[a] The plus signs indicate the degree of inhibition effect of the antibiotic.

The antibiotic substance in the broth is stable for a long time both at room temperature and under refrigeration. It is stable at acid pH and less stable at alkaline pH. It is quite stable up to 80° and gets partially inactivated at 100°. Copper ions have no effect on it. It is toxic; 100 mg. substance per Kg. body weight of rat, kills the rat when given intraperitoneally.

Structure of the Antibiotic Molecule.—The molecular weight is 256 (Rast method). It contains carbon, hydrogen, and oxygen, but nitrogen, halogens, and phosphorus are absent. The product decolorized acidified potassium permanganate solution and bromine in carbon tetrachloride. It gave a brown color with aqueous ferric chloride and deep blue color (first brown) with sodium nitrite in concentrated sulfuric acid, indicating the presence of phenolic group in the molecule. Carboxyl group was detected by the evolution of carbon dioxide when a few crystals of the substance were added to a solution of sodium bicarbonate.

The physicochemical and antibacterial properties of the compound compared favorably with those of citrinin described (3–5). Both of them have the same functional groups, i. e., phenolic and carboxyl, both are active against *V. cholera* and Gram-positive organisms, and activities of both are completely destroyed by cysteine (6). It was therefore concluded that the new antibiotic might be identical with citrinin. Paper chromatographic studies with the authentic sample of citrinin as a standard (7) and color test for citrinin as described by Tauber, *et al.* (8), showed that the antibiotic is citrinin. In the chromatographic studies, the antibiotics were located by the bioautographic technique using *B. subtilis* as the test organism. It failed to depress the m.p., 165–169°, of the authentic specimen on admixture with it.

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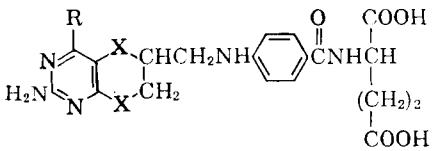
Communications

Analogs of Tetrahydrofolic Acid VI

N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid, an Inhibitor of Folic Reductase

Sir:

Fifteen enzymes utilizing folic acid, tetrahydrofolic acid (I), or derivatives of tetrahydrofolic acid are known (1–3). A number of these enzymes are inhibited by aminopterin (4-amino-4-deoxyfolic acid) (4–7), but nearly as many are not (6, 8–10). 5,6,7,8-Tetrahydroaminopterin (II) can inhibit some of the enzymes not inhibited by aminopterin (10, 11). 5,8-Dideaza-5,6,7,8-tetrahydroaminopterin (IV) has been recently synthesized (12) and found to have inhibitory properties similar to tetrahydroaminopterin (12, 19). In addition, 5,8-dideaza-5,6,7,8-tetrahydrofolic acid (III) (13) has been found to bind to folic reductase eight times stronger than the substrate, folic acid (14).



I. R = OH, X = —NH—
II. R = NH_2, X = —NH—
III. R = OH, X = —CH_2—
IV. R = NH_2, X = —CH_2—

The folic cofactor area should be a prime target for utilization of recent developments in nonclassical antimetabolite theory (15–17) since larger differential effects on inhibition of these enzymes might be obtained by the bulk principle of specificity (15), the exo-alkylating irreversible inhibition phenomenon (16), and the bridge principle of specificity (17). In order to use

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The physicochemical and antibacterial properties of the compound compared favorably with those of citrinin described (3–5). Both of them have the same functional groups, i. e., phenolic and carboxyl, both are active against *V. cholera* and Gram-positive organisms, and activities of both are completely destroyed by cysteine (6). It was therefore concluded that the new antibiotic might be identical with citrinin. Paper chromatographic studies with the authentic sample of citrinin as a standard (7) and color test for citrinin as described by Tauber, *et al.* (8), showed that the antibiotic is citrinin. In the chromatographic studies, the antibiotics were located by the bioautographic technique using *B. subtilis* as the test organism. It failed to depress the m.p., 165–169°, of the authentic specimen on admixture with it.

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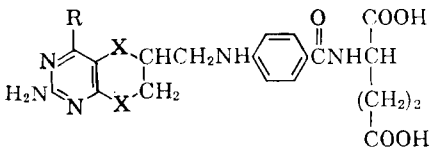
Communications

Analogs of Tetrahydrofolic Acid VI

N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid, an Inhibitor of Folic Reductase

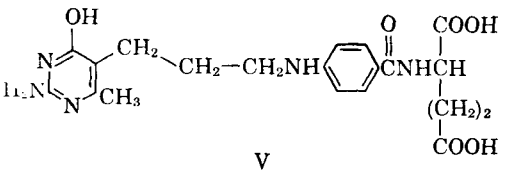
Sir:

Fifteen enzymes utilizing folic acid, tetrahydrofolic acid (I), or derivatives of tetrahydrofolic acid are known (1–3). A number of these enzymes are inhibited by aminopterin (4-amino-4-deoxyfolic acid) (4–7), but nearly as many are not (6, 8–10). 5,6,7,8-Tetrahydroaminopterin (II) can inhibit some of the enzymes not inhibited by aminopterin (10, 11). 5,8-Dideaza-5,6,7,8-tetrahydroaminopterin (IV) has been recently synthesized (12) and found to have inhibitory properties similar to tetrahydroaminopterin (12, 19). In addition, 5,8-dideaza-5,6,7,8-tetrahydrofolic acid (III) (13) has been found to bind to folic reductase eight times stronger than the substrate, folic acid (14).

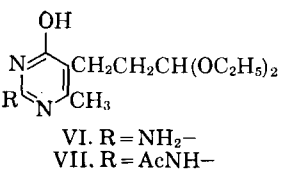


I. R = OH, X = —NH—
II. R = NH_2, X = —NH—
III. R = OH, X = —CH_2—
IV. R = NH_2, X = —CH_2—

The folic cofactor area should be a prime target for utilization of recent developments in nonclassical antimetabolite theory (15–17) since larger differential effects on inhibition of these enzymes might be obtained by the bulk principle of specificity (15), the exo-alkylating irreversible inhibition phenomenon (16), and the bridge principle of specificity (17). In order to use



V



VI. R = NH_2–
VII. R = AcNH–

these three corollaries of nonclassical antimetabolite theory, it would be advisable to have an inhibitor that can be made by a relatively short sequence and the sequence should be one that lends itself to the placing of substituents in a variety of positions. Synthesis of compound V, which satisfies both the inhibitor and synthetic requirements, is the subject of this communication.

Ethyl 2-acetylglutaraldehydate, prepared in 47% yield from Michael addition of ethyl acetoacetate to acrolein (18), was converted in boiling ethanolic ammonium chloride in 63% yield to its diethyl acetal (VIII), b.p. 110–112° (0.2 mm.).

Anal.—Calcd. for $C_{13}H_{24}O_5$: C, 60.1; H, 9.64. Found: C, 60.0; H, 9.70.

Reaction of the keto ester acetal (VIII) with guanidine in boiling absolute ethanol afforded a 76% yield of VI, m.p. 179–180°.

Anal.—Calcd. for $C_{12}H_{21}N_3O_3$: C, 56.6; H, 8.31; N, 16.5. Found: C, 56.5; H, 8.10; N, 16.7.

Treatment of VI with acetic anhydride in pyridine at 85° gave a 57% yield of VII, m.p. 150°.

Anal.—Calcd. for $C_{14}H_{23}N_3O_4$: C, 56.6; H 7.81; N, 14.1. Found: C, 56.8; H, 7.94; N, 14.3.

Hydrolysis of VII by short boiling in water afforded 46% of 2-acetamido-4-hydroxy-6-methyl-5-pyrimidinepropionaldehyde (IX), m.p 159–160°.

Anal.—Calcd. for $C_{10}H_{13}N_3O_3$: C, 53.8; H, 5.87; N, 18.8. Found: C, 53.6; H, 5.95; N, 18.6.

Condensation of the pyrimidinepropionaldehyde (IX) with *p*-aminobenzoyl-L-glutamic acid in boiling alcohol, reduction of the resultant anil (X) with sodium borohydride, and basic hydrolysis of the N-acetyl group gave, after full purification, 15% of V as a white solid,[1] m.p. >250°; $\lambda_{max.}^{pH\ 1}$ 222 (ε 27,800), 270 (ε 19,200), and 303 mμ (ε 11,400); $\lambda_{max.}^{pH\ 8.4}$ 295 mμ (ε 15,100); $\lambda_{max.}^{pH\ 13}$ 284 mμ (ε 20,500).

Anal.—Calcd. for $C_{20}H_{23}N_5O_6$: C, 55.8; H, 5.85; N, 16.3. Found: C, 56.2; H, 5.66; N, 16.5.

Compound V inhibited (14) folic reductase with $K_i = 7 \times 10^{-6}$, about the same as the K_m of folic acid. Since this compound is constructed from ethyl acetoacetate, acrolein, guanidine, and *p*-aminobenzoyl-L-glutamic acid, it is obvious that a variety of derivatives of V can be made by modifying the four components or by transformations of VI; in this way, compounds could be obtained that, by use of nonclassical antimetabolite theory, might selectively inhibit some of the 15 enzymes in the folic acid cofactor area. In addition, considerable information could be obtained about the relative binding and conformational requirements of these substrates to their respective enzymes (19). Such a program is continuing in these laboratories.

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[1] The final product contained about 1% of the anil (X) or *p*-aminobenzoyl-L-glutamic acid as quantitatively determined by the Bratton-Marshall test; the crude yield was 61% of 73% purity.

Received March 8, 1962.
Accepted for publication April 6, 1962.
This work was generously supported by Grant CY-5845 of the National Cancer Institute, U. S. Public Health Service.

Chamazulene from *Artemesia caruthii*

Sir:

In a recent survey of terpene-bearing plants of the Sonoran desert, it was reported (1) that the steam distillate of common sagebrush (*Artemesia caruthii* Wood var. *wrightii* A. Gray) contained a blue oil. Because of the widespread interest (2*a*, 2*b*) in the azulenes of the Compositae family, and in particular the Artemesia species, we undertook to identify the blue component of this oil. It has now been characterized as chamazulene (1,4-dimethyl-7-ethylazulene).

Stems, leaves, and flowers of *A. caruthii* were collected in August 1961 at elevations above 6000 ft. in the Graham Mountains of Arizona. The wet plant material was steam distilled; the blue distillate was taken up in petroleum ether and extracted into 85% phosphoric acid. The concentrated acid solution was diluted with water, regenerating the blue oil, which was then partitioned into petroleum ether again. After being dried over magnesium sulfate, the petroleum ether solution was evaporated to dryness under vacuum. A viscous blue oil remained; from 1.0 Kg. of wet plant material, 0.50 Gm. of azulenic compound was obtained.

After further purification by chromatography over alumina, the oil was converted to a trinitrobenzene addition compound; m.p. 130.5–131.0°, uncor. [Lit. (3), 132° for chamazulene TNB compound].

Anal.—Calcd. for $C_{14}H_{16}.C_6H_3(NO_2)_3$: C, 60.45; H, 4.82. Found: C, 60.35; H, 4.77.

The ultraviolet, visible, and infrared spectra were identical to the reported values (4) for chamazulene. As might be expected, these spectra were almost superimposable on those of an authentic sample of guaiazulene (1,4-dimethyl-7-isopropylazulene) measured in this laboratory. However, the critical data for the guaiazulene TNB compound [m.p. 151–152° (4); anal. calcd.: C, 61.31; H, 5.15] clearly differentiate it from the chamazulene compound.

Only a few plant species yield chamazulene on simple steam distillation. One of these is *Artemesia absynthium* (5). The precursors of this blue oil have been shown to be a group of closely-related sesquiterpene lactones (5). Because of the recent scientific interest in these sesquiterpene compounds, as well as local interest in the medicine and taxonomy of the Artemesia species in southwestern United States, we are now engaged in isolating and characterizing the lactones of *A. caruthii*.

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Accepted for publication April 12, 1962.
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Screening Antifatigue Agents by Radiorespirometry

Sir:

Over the past 5 years a number of publications have appeared on the use of the potassium and magnesium salts of aspartic acid in the treatment of human fatigue (1–3). To measure the effect of various agents on fatigue in animals, the rat swim test has been found useful (4). Ideally, the mechanisms whereby these substances modify the energy-yielding processes in fatigue should be studied in the exercising animal. However, the many technical difficulties this would present led us to develop instead the following assay based on the utilization of glucose, a primary source of energy, during recovery from fatigue.

Male white rats, weighing 280 to 350 Gm. were burdened with lead amounting to 3% of their body weight and permitted to swim in tanks containing 18 inches of water at 28° until no longer able to surface.

Prior treatment consisted of 30 mg. per Kg. oral doses, beginning with a single dose 2 days before the experiment, two doses on the day before, and one or two on the day of the experiment, depending on whether the animal was to be used in the morning or afternoon, respectively. The last dose was given immediately before the animal was placed in the tank. Upon removal

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TABLE I.—EFFECT OF ASPARTATE SALTS ON RESPIRATORY $C^{14}O_2$ PRODUCTION

Treatment	No. of Animals	Respired CO_2[a] 30 min.	60 min.	90 min.
Rested controls	5	2.18 ± 0.44	2.36 ± 0.62	1.75 ± 0.40
Swum controls	15	0.36 ± 0.15	0.62 ± 0.11	0.62 ± 0.25
K Asp. 30 mg./Kg.[b]	8	0.58 ± 0.32	0.96 ± 0.54	0.71 ± 0.25
P[c]		0.025	<0.025, >0.01	0.20
Mg Asp. 30 mg./Kg.	6	0.47 ± 0.32	0.66 ± 0.36	0.58 ± 0.22
P		0.15	0.35	...
K Asp. 15 mg./Kg. + Mg Asp. 15 mg./Kg.[d]	9	0.65 ± 0.40	0.91 ± 0.40	0.87 ± 0.18
P		0.05	<0.025, >0.01	<0.025, >0.01

[a] Means and standard deviations in $\mu c. \times 10^{-3}$ registered at the times indicated after injection of C^{14}-glucose. [b] Aspartate salts were given orally. [c] Significance of mean increase compared with swum controls by one-tailed *t*-test. [d] Spartase, Wyeth.

from the tank (discarding those which did not swim at least 15 minutes) 0.4 ml. of a solution containing 1.7 $\mu c.$ per ml. of uniformly labeled C^{14}-glucose in 2% nonradioactive glucose was injected into the external saphenous vein and the rat was placed in a closed glass chamber swept by 140 ml. of air per minute. The C^{14} content of the respired carbon dioxide was measured by passing the effluent air through the 250-ml. ionization chamber of a Nuclear-Chicago "Dynacon" electrometer connected to a 1 ma. linear recorder.

Representative data from the respired $C^{14}O_2$ curves for the controls and animals receiving individual and mixed aspartate salts are tabulated in Table I. The mean values for the control animals and those receiving the mixed salt are illustrated in Fig. 1, which demonstrates the essential factors observed in these experiments: (*a*) the length of the induction period before appreciable $C^{14}O_2$ is excreted (considerably prolonged in fatigued animals as compared with rested controls); (*b*) the considerably lower maximal rate of $C^{14}O_2$ evolution in the fatigued animals and the longer time taken to reach the maximal rate; (*c*) the differences in total $C^{14}O_2$ output as indicated by the areas under the curves; (*d*) the elevation of the curves of the treated animals above those of the fatigued controls.

Studies on the method are being continued in view of the fairly high degree of individual variation encountered, and glucose-1-C^{14} and glucose-6-C^{14} are being tried in the hope that one of these will result in a more specific response and also help to elucidate the mechanism.

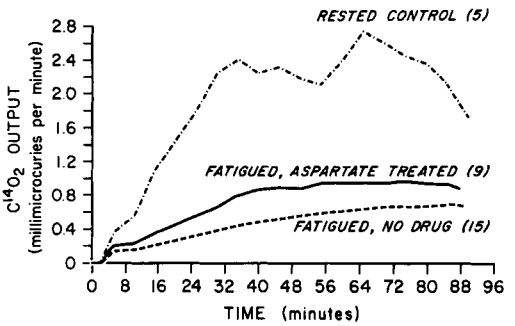


Fig. 1—Respiratory $C^{14}O_2$ output following C^{14}-glucose injection.

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Received March 5, 1962.
Accepted for publication March 27, 1962.
The authors are grateful to Miss Anne Gillen for the statistical analyses.

l-N-Norarmepavine

Sir:

Recent reports have described the isolation from Asiatic lotus, *Nelumbo nucifera* Gaertn. (*Nymphaeaceae*), of the alkaloids nuciferine (1, 2, 4), roemerine (2, 4), nornuciferine (2–4), and *dl*-armepavine (5). We report herewith the isolation from American lotus, *Nelumbo lutea* (Willd.) Pers. (*Nymphaeaceae*)[1] of the alkaloids nuciferine (0.046%), *dl*-armepavine (0.0046%), and an apparently new alkaloid (0.047%), to which we assign structure I and the name *l*-N-norarmepavine.

The new alkaloid, m.p. 152–153°, $[\alpha]_D^{25}$ − 23°

[1] Air-dried leaves and stems, collected in Wisconsin in 1959–1961. We thank Professor H. H. Iltis of the University of Wisconsin for confirming the identity of the plant. A voucher specimen is deposited in the University of Wisconsin Herbarium.

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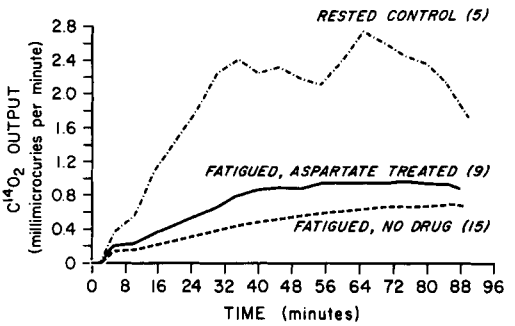


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Accepted for publication March 27, 1962.
The authors are grateful to Miss Anne Gillen for the statistical analyses.

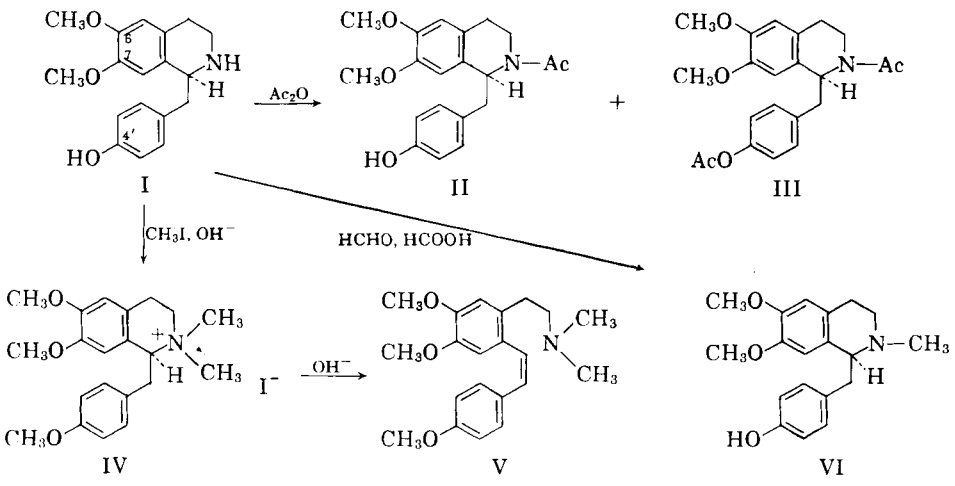
l-N-Norarmepavine

Sir:

Recent reports have described the isolation from Asiatic lotus, *Nelumbo nucifera* Gaertn. (*Nymphaeaceae*), of the alkaloids nuciferine (1, 2, 4), roemerine (2, 4), nornuciferine (2–4), and *dl*-armepavine (5). We report herewith the isolation from American lotus, *Nelumbo lutea* (Willd.) Pers. (*Nymphaeaceae*)[1] of the alkaloids nuciferine (0.046%), *dl*-armepavine (0.0046%), and an apparently new alkaloid (0.047%), to which we assign structure I and the name *l*-N-norarmepavine.

The new alkaloid, m.p. 152–153°, $[\alpha]_D^{25}$ − 23°

[1] Air-dried leaves and stems, collected in Wisconsin in 1959–1961. We thank Professor H. H. Iltis of the University of Wisconsin for confirming the identity of the plant. A voucher specimen is deposited in the University of Wisconsin Herbarium.



(c 1.33, $CHCl_3$), $[\alpha]_{546}^{26}$ − 40° (c 1.03, $CHCl_3$), λ_{max}^{EtOH} 228 mμ (ε 15,400), 282 mμ (ε 5,000), 287 mμ (ε 4,900), was shown to have the formula $C_{16}H_{15}NO(OCH_3)_2$.

Anal.—Calcd. for $C_{18}H_{21}NO_3$: C, 72.21, H, 7.07; N, 4.68; OCH_3, 20.73. Found: C, 72.06; H, 7.02; N, 4.89; OCH_3, 20.15; equiv. weight by titration with perchloric acid, 304.

Acetylation of I with acetic anhydride-pyridine, followed by chromatography on neutral alumina, gave an amorphous O,N-diacetate (III), IR bands at 5.70, 6.18 μ, and a crystalline N-acetate (II), m.p. 237–238°, IR band at 6.20 μ but none in the 5.6–6.0 μ region, soluble in dilute sodium hydroxide.

Anal.—Calcd. for $C_{20}H_{23}NO_4$: C, 70.36; H, 6.79; N, 4.10. Found: C, 69.95; H, 6.82; N, 3.92.

The foregoing facts established that I possesses a phenolic hydroxyl group and an acylable amino group.

Exhaustive methylation of I gave the 1-(4′-methoxybenzyl) - 6,7 - dimethoxy - 2 - methyl-1,2,3,4-tetrahydroisoquinoline methiodide (IV), m.p. 127–129° (6–9). Treatment with alkali yielded the methine V, m.p. 86–87°, characterized by direct comparison (mixed m.p., IR) with an authentic sample (10). Methylation of I with formalin and formic acid yielded *d*-armepavine (VI), m.p. 139–140°, $[\alpha]_D^{25}$ + 91° (c 1.19, $CHCl_3$). The infrared spectrum of the methylation product in chloroform was superimposable upon that of a sample of *l*-armepavine, m.p. 140–141°, prepared by sodium-liquid ammonia reduction of cycleanine (11). Mixture of equal quantities of *d*- and *l*-armepavine and crystallization from acetone yielded *dl*-armepavine, m.p. 166–167°. The melting point was undepressed by admixture of the sample isolated from *N. lutea*, and the infrared spectra of the respective samples in chloroform were identical. Hence, the name *l*-N-norarmepavine is proposed for the new phenolic secondary base. A total synthesis of *dl*-1-(4′-hydroxybenzyl) - 6,7 - dimethoxy - 1,2,3,4 - tetrahydroisoquinoline has been reported (12).

Diazomethane methylation of *d*-armepavine (derived from *l*-N-norarmepavine) yielded a nonphenolic base, m.p. 62–63°, $[\alpha]_D^{28}$ + 79° (c 0.84, $CHCl_3$) which was identified as *d*-l-(4′-methoxybenzyl) - 6,7 - dimethoxy - 2 - methyl - 1,2,3,4-tetrahydroisoquinoline by direct comparison with an authentic sample (10). The latter compound has recently been shown to possess the L-configuration at C-1 (13).[2] Consequently, the absolute configuration of L-(*l*)-N-norarmepavine may be represented as in I.

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Received March 26, 1962.
Accepted for publication April 11, 1962.
This investigation was supported in part by a grant (H-2952) from the National Institutes of Health.

[2] We thank Professor Tomita cordially for informing us of results prior to publication.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

July 1962 volume 51, number 7

Review Article

Crystallography. Part II

By JOHN A. BILES

OPTICAL CRYSTALLOGRAPHY

Optical Indicatrix

The manner in which light is affected by crystals arises from the internal structure. Crystals are placed in one of two classes depending on the effect of the transmission of light in different directions through the crystals. These classes are isotropic and anisotropic. Fletcher (192) introduced the optical indicatrix to explain the effect of light when passing through a nonopaque substance. In relation to this, Wooster (193) has discussed the geometrical properties of a triaxial ellipsoid.

To visualize the optical indicatrix one can consider a point source of light in the center of a crystal. Allow the light to propagate in every direction of the crystal. After an elapse of time, draw wave fronts for all of the waves. The result of all of the wave fronts is either a sphere, spheroid, or ellipsoid (triaxial). The sphere is termed an isoaxial indicatrix; solids producing such a figure are termed isotropic. These solids are also termed isometric. The spheroid, either prolate or oblate, is termed a uniaxial indicatrix; solids producing such a figure are termed anisotropic and belong to the tetragonal and hexagonal systems. The triaxial ellipsoid is termed a biaxial indicatrix; solids producing such a figure are termed anisotropic and belong to the orthorhombic, monoclinic, and triclinic crystal systems.

In order to determine the orientation of the optical indicatrix within the crystal, the crystallographer will examine the interference figures. These figures are defined as telescopic images of convergent light passing through anisotropic crystals between crossed nicols. The theory of formation of interference figures has been proposed by Kamb (194).

Refractive Index and Molar Refraction

The velocity of the light on passing through the crystal is determined by measuring the refractive index of the section of the crystal viewed. Meaningful refractive indexes are termed principal indexes. The principal indexes are determined by viewing sections which lie in the planes of the indicatrix coordinates. This is done by looking for centered interference figures.

The refractive index depends on the velocity of the light which, in turn, depends upon the atoms, ions, or aggregates present and their position occupied in the lattice. This index is proportional to the polarizability of the molecule. The ease of displacement of positive and negative charges with respect to each other in an electric field is called polarizability. Thus, the refractive index is governed by the kind of atoms present in the molecule and the packing of the molecules, atoms, or ions in the crystalline state. The polarizability is the same in any direction in the crystal for isotropic solids. The polarizability will vary with the direction of propagation of light in anisotropic crystals. The net effect is the polarization of light which is

Received from the University of Southern California, Los Angeles 7.

Editor's note: Part 1 of this review appeared in the June issue; THIS JOURNAL, 51, 499(1962).

quite evident when anisotropic crystals are examined under the microscope between crossed nicols.

It has been shown that the mathematical relationship of the total induced polarization which is caused by the distortion of the electron shells in the molecule is equivalent to the molar refraction (195, 196). Brühl found that conjugated unsaturated groups caused an increase of the molecular refractive and dispersive power (197). This optical exhaltation was quantitative and dependent on the nature and number of unsaturated atomic groups.

Bragg (198) was able to calculate the refractive index values for calcite and aragonite utilizing the specific refractions of the carbonate and calcium ions. This calculation was aided with an X-ray analysis which showed that carbonate ions were flat and the planes of the ions normal to the direction of the least refractive index. Under the influence of an electric field an atom develops a doublet of moment *se*, corresponding to the relative displacement to a distance *s* of elementary charges $\pm e$

$$s = E'e\lambda \qquad \text{(Eq. 3)}$$

where E' = strength of field in neighborhood of atom and λ = characteristic constant of atom. This does not depend on the direction of the local field if isotropic.

In each ml. of polarized medium, let there be N_1 atoms which form doublets of moments, e, N_2 forms doublets of moment s_2e, etc. It can be shown that

$$(K - 1)\bar{E} = P = (N_1s_1e + N_2s_2e + \ldots) \qquad \text{(Eq. 4)}$$

where $\bar{E}$ = average value of electric density throughout medium, P = total polarization per unit volume, and K = dielectric constant of the medium.

The strength of a doublet is

$$s_1/\lambda_1 \qquad \text{(Eq. 5)}$$

Strength of the doublet is also

$$(\bar{E} + {}^1/_3P)e \qquad \text{(Eq. 6)}$$

Thus

$$K - 1 = \frac{P}{\bar{E}} = \frac{\Sigma Ne^2\lambda}{1 - {}^1/_3 \Sigma Ne^2\lambda} \qquad \text{(Eq. 7)}$$

$$K = n^2 \qquad \text{(Eq. 8)}$$

$$n^2 - 1 = \frac{\Sigma Ne^2\lambda}{1 - {}^1/_3 \Sigma Ne^2\lambda} \qquad \text{(Eq 9)}$$

Or

$$\frac{n^2 - 1}{n^2 + 2} = {}^1/_3 \Sigma Ne^2\lambda \qquad \text{(Eq. 10)}$$

If both sides of Eq. 10 are multiplied by M/ρ

$$\frac{M}{\rho} \cdot \frac{n^2 - 1}{n^2 + 2} = a\,({}^1/_3 N_0e^2\lambda_1) + b\,({}^1/_3 N_0e^2\lambda_2) + \ldots \qquad \text{(Eq. 11)}$$

This additive law is obeyed approximately for isotropic material. The law would be exactly obeyed were it not for the fact that all other atoms around any given atom are not arranged at random, since those which form part of the same molecule have a definite relationship to it.

Bragg considered the polarization of the ions in the three directions; he also considered that the polarization in the y and z directions affected the polarization in the x direction. Allowance for these effects yielded an equation for the refraction index, n, for any given direction of the electric vector as

$$n^2 - 1 = \frac{(c_1N_1e^2\lambda_1 + c_2N_2e^2\lambda + \ldots)}{1 - {}^1/_3(c_1N_1e^2\lambda_1 + c_2N_2e^2\lambda_2 + \ldots)} \qquad \text{(Eq. 12)}$$

The polarization of the calcium ion in calcite or aragonite is not too important since the ion contributes about 15% of the total refractivity. The value for the calcium ion is given as 1.99. The value for the calcium in calcite and aragonite is determined with the knowledge of the density of the solid. Thus

$$Ne^2\lambda = \frac{3\rho}{M} I_{Ca^{++}} \qquad \text{(Eq. 13)}$$

The value for Eq. 13 is 0.165 for calcite and 0.175 for aragonite. The I value for carbonate was determined utilizing the molecular refractions of Al_2O_3, NO_3^-, CO_3^-, and SO_3. The I_0 for the three oxygens is between 3.3 and 3.7. When the I value for $CO_3^=$ is substituted into Eq. 13, the value of 0.822 is obtained for calcite and 0.873 for aragonite. The value for e^2 may be calculated and then substituted back into Eq. 9. When corrections were made for neighboring interactions, Bragg found that the observed and calculated refractive indexes were similar. These values are reported in Table IV.

TABLE IV.—CALCULATED AND OBSERVED REFRACTIVE INDEX VALUES FOR CALCITE AND ARAGONITE

Calcite	Calcd.	Observed
ϵ	1.488	1.486
ω	1.631	1.658
Aragonite		
α	1.538	1.530
β	1.694	1.681
γ	1.680	1.686

It is to be noted from the data recorded in Table IV that the calculated value for beta in aragonite is greater than the value for gamma. Those who have a knowledge of the biaxial indicatrix know that this is impossible. It does indicate, however, that at an early date there was developed a quantitative relationship for the calculation of refractive indexes of solids.

Bragg also pointed out that the oxygens surrounding the sulfur in the sulfate ion were uniformly distributed in the three-dimensional pattern. He thus stated that this tetrahedral arrangement of oxygen atoms was the cause of the small difference in the principal refractive indexes. This does point to the use of refractive indexes or molar refractions in prediction of coordination complexes. Dachille and Roy used infrared absorption and molar refractions to check coordination (199). The investigators related primary coordination, atomic number, atomic weight, and ionic charge to absorption wavelengths for the major bands. For AX_2 compounds, a K value of 0.168 was obtained where K was found to be

$$K = \frac{(\text{C.N.})(\mu)(A_A + A_X)^{1/3}}{Z_A Z_Z \cdot \lambda^2} = 0.168 \quad \text{(Eq. 14)}$$

where C. N. = coordination number, $Z_A Z_Z$ = valence, μ = reduced mass term, $A_A + A_X$ = sum of atomic numbers, and λ = main absorption wavelength in microns.

Equation 14 was applied to ABO_4 type compounds and the K value did not agree for the different compounds studied as well as the value agreed for the AX_2 compounds. The molar reactivities supported the conclusions reached using the infrared data.

Batsanov (200) calculated the electronic polarizability of atoms with intermediate bond character. The determination of the effect of the change of bond polarity on the atomic refraction was discussed. The study was quantitated by comparing the ionic and atomic refractivities of the elements. After finding the change in the atomic size with changing ionization and by using the proportionality of refractions to atomic volumes, a law was proposed which governs the change in the electronic polarizabilities of the atoms with a change in the bond polarity. Nutt (201) has reported a correlation and prediction of the optical and thermodynamic properties of saturated liquid hydrocarbons by the group contribution method. Such a relationship can be applied to the solid hydrocarbons which are nonpolar without too much error. Nutt used the Sellmeier-Drude equation for calculations.

The molar refractions of molecules in the solid state have been experimentally determined and compared with the calculated values. When reports were submitted for "Crystallographic Data," molar refraction values were included (202). The molar refractions have been experimentally determined for the antihistamines (203), sulfonamides (204), antitubercular compounds (205), and steroids (206). The experimental values were in close agreement with the calculated values. The reader should recall that Bragg, in calculating the refractive indexes of calcite and aragonite, stated that experimental and calculated molar refractions could be identical were it not for the fact that all other atoms around any given atom are not arranged at random (198). In some cases the experimental molar refraction showed a negative deviation from the calculated value; the deviation was by as much as 14%. Compounds showing such deviation included thiamine hydrochloride (207), α-pyridine sulfonic acid (208), cyclotrimethylenetrinitramine (209), cyclotetramethylenetetranitramine (210), and streptidine sulfate monohydrate (211). On the other hand, it was observed that some highly conjugated systems showed a positive deviation from the calculated molar refraction. This deviation occurred with *trans*-azobenzene (212), *trans*-stilbene (213), *trans*-diethylstilbestrol (214), and 1,3,5-tri(*p*-chlorophenyl)benzene (215). The positive deviation was shown also by 4-aminosalicylic acid (216), vanillin (217), 2-mercaptobenzothiazole (218), gliotoxin (219), and 2-naphthoic acid (220). The observed and calculated molar refraction for *l*-naphthoic acid agreed within 1% (221).

Brasseur applied the Lorentz and Lorenz equation to the study of inorganic materials (222). The results obtained were satisfactory for the carbonates of calcium, magnesium, manganese, zinc, ferrous, barium, strontium, and lead.

A comparison between the square of the geometric mean refractive index of crystals and the dielectric constant determined in solvents can be made. Maxwell derived a relationship of the refractive index for light of long wavelength to the dielectric constant (223). This relationship is given by Eq. 8. This relationship is close when the molecules are far apart. A comparison of the dielectric constant calculated from refractive indexes of compounds in the solid state to the dielectric constant of the molecule in solution is shown in Table V. The dielectric constant of acetanilid and D.D.T. was determined using different solvents, and the value changed with each solvent. In addition, D.D.T. was liquefied and the dielectric constant was deter-

TABLE V.—COMPARISON OF THE DIELECTRIC CONSTANTS OF SOME DRUGS OBSERVED BY DIFFERENT METHODS (224–229)

Compound	ϵ (from Dioxane)	n^2
Acetanilid	3.618	2.616
	2.432[a]	...
Acetylsalicylic acid	2.2583	2.583
Barbital[b]	2.2556	2.373
	...	2.322
	...	2.338
Cholesterol	2.2134	2.369
D.D.T.[b]	2.2614	2.773
	2.393[c]	2.752
	2.2387[a]	...
	2.900[d]	...
	2.381[e]	...
Phenobarbital[b]	2.2477	2.605
	...	2.525
	...	2.601

[a] From benzene. [b] Polymorphic. [c] From carbon tetrachloride. [d] Pure liquid at 104°. [e] Pure liquid at 145°.

mined at different temperatures. The values for two such temperatures are included in the table.

Kleber (230) reported the relation between the dielectric constant and the structure of a crystal. The relation was based on the polarizability of the lattice, the anisotropic nature of the lattice, and the presence of dipoles in the crystal lattice. He related the dielectric constant to the number of structural units on 1 ml. of the lattice and the molar polarizability by

$$\frac{\epsilon - 1}{\epsilon + 2} \cdot \text{v.} = \frac{4\mu}{3} N \left[\alpha \frac{\mu^2}{3kt}\right] \quad \text{(Eq. 15)}$$

Pulou (231) measured the specific inductive capacities for 60 crystals. The dielectric constant of the crystal was measured relative to the characteristic cleavage plane or optic axis. McMahon (232) noted that the dielectric constant of certain organic compounds became higher than normal when solidified in either alternating or undirectional electric fields. In a magnetic field an anisotropic change resulted in an increase in the direction of applied field and decreased perpendicular to the field. Allen (233) related the refractive index to the specific gravity for some crystalline compounds. He stated that the geometric mean of the three principal refractive indexes was proportional to the specific gravity as given by

$$k = \frac{\alpha\beta\gamma}{d} \quad \text{(Eq. 16)}$$

He reported that his equation was good for some polymorphs but not others.

The refractive index has also been used in establishing the conformation of cyclohexane derivatives (234–236). *Cis-trans* pairs of disubstituted cyclohexanes can be differentiated by measuring the refractive index of the melt. The isomer having the higher index of refraction and higher density would be the isomer with the less conformational stability. Kelly (237) proposed that for isomeric cyclohexanes and tetrahydropyran derivatives similarly substituted on corresponding ring carbon atoms, the refractive indexes and densities increase with increasing number of axial substituents.

The refractive index of solids has been used for the identification of mixtures (238), and as a supplement to X-ray analysis of solid solutions to detect any possible heterogeneity (239). The difference between the highest and lowest refractive index values, the birefringence, has been used to predict molecular orientations (240), specific strength of cotton fibers (238), and the nature of montmorillonite complexes (241). In studying the birefringence of aromatic complexes of clays, it was noted that the polarizability of the aromatic molecule would affect the overall polarizability of the complex. When the aromatic nucleus complexed parallel to the silicate sheet, the negative birefringence increased.

Chemical Microscopy and Optical Crystallographic Properties

The development of chemical microscopy and optical crystallography in the United States was largely the result of the works of Chamot and Mason, Keenan, McCrone, and Witt and Poe. Review articles on chemical microscopy have appeared periodically (242–249). Mason has justified chemical microscopy in the technical organization (250). Hartshorne has discussed the value of optical crystallography in industry (238). During the first and middle part of the first half of the twentieth century there was no standard practice in reporting crystallographic properties. As a result, recommended practices were published (251). Through the efforts of McCrone, optical crystallographic data were published monthly beginning in 1948 (202). This practice ceased in *Analytical Chemistry* with the report of alpha-D-talose (252). Similar reports are now appearing in THIS JOURNAL (253). Winchell has tabulated the optical properties for many compounds (224) and at the peak of the interest in optical crystallographic properties, Kirkpatrick suggested punched cards for the filing of such information (254). McCrone also suggested a punched card system (255).

Keenan and his workers contributed a large amount of data to the literature. Largely through his efforts, data were collected on U.S.P. XII compounds (225). Optical crystallographic

properties were determined for N.F. X compounds (256). Witt and Poe and co-workers contributed to the development of optical crystallographic properties of organic compounds of pharmaceutical interest (203–205,257–260). Plein and Dewey have contributed particularly in the identification of amines (261–264). The optical crystallographic studies of Bryant and Mitchell remain a classic (265–269). Bryant prepared the *p*-bromanilides of acetic acid and propionic acid and found that the acids could be semiquantitated in binary mixtures by determining the optic axial angles using five monochromatic radiations of the mercury arc. With the aid of dispersion of the optic axes and the melting points, the weights of the components could be calculated. Their additional reports emphasized various types of dispersion including crossed axial plane, inclined, horizontal, crossed, and ellispoidal dispersion. In reporting optical crystallographic data of different compounds, McCrone and co-workers had to resort to some imaginative work. Deffner and McCrone (270) reported the problems involved in the determination of the refractive indexes of *p*-dimethylaminobenzaldehyde since the compound was soluble in all of the refractive index oils. McCrone found polymorphism to be prevalent with some of the compounds tested. The relationship between temperature and transition was reported for salicylamide (271). Rose, Van Camp, and Williams have contributed to the literature. Their studies include the Rauwolfia alkaloids (272–276), alkaloids from *Vinca rosea* (277), antibiotics (278, 279), and other compounds of pharmaceutical interest. Shell (280, 281) and Clarke and Krc (282) have contributed also. Shell refined some of his studies by utilizing the universal stage (203, 204). He also stated that the orientation of the biaxial indicatrix in triclinic crystals could be determined only with the use of the universal stage (283).

The work of Prien and Frondel should not go unnoticed by crystallographers. Their original work was important in solving the toxicology of sulfonamide therapy. Through the use of optical crystallographic properties, they were able to prove that acetylsulfathiazole crystallized in the kidney. They also studied the acetyl derivatives of sulfanilamide and sulfapyridine (284–286). These investigators continued their studies with an emphasis on urinary calculi (287–289). Prien reported the structure and comparison of some 6000 human urinary calculi studied by crystallographic techniques. He was able to correlate the optical crystallographic studies with pathogenesis, and found that it was often possible to reconstruct the clinical history by an optical study of the calculus obtained from the patient. In a paper concerning the deposition of solids in the body, Pfeiffer discussed the use of X-ray diffraction, optical crystallography, and infrared absorption spectrophotometry (290). Pfeiffer suggested that the physical chemist could devote greater time to help solve the problem of cholesterol deposition. Stewart made an attempt to examine atherosclerotic and normal tissues and plasma in the fresh state and at temperatures up to 37° with the thought that results might have some bearing on the problem of the integration of certain forms of lipid in tissue. Stewart discussed the significance of the birefringent material observed (291).

Optical crystallographic properties have been used to aid in predicting molecular orientations in the solid state. Studies have been published for purines and pyrimidines (260), and dicarboxylic acids (292, 293). The limitations of the use of optical crystallographic properties in helping to determine molecular orientations has been discussed by Krishnan and Lonsdale and coworkers (294, 295).

Fusion Methods

The Kofler group has been responsible for the development of "Thermo-Mikro-Methoden" (296). L. Kofler, holder of the Magister in Pharmacy, Ph.D., and M.D., was a pharmacognosist but had to turn to microscopy because of lack of facilities. His wife, Adelheid, was a mineralogist who received an M.D. (297). McCrone and co-workers have been responsible for the development of fusion methods in the United States (298). McCrone pointed out that both methods were influenced by publications of Otto Lehmann. The two methods were developed apparently independently (298). Reports utilizing Koflers' procedures continue to be published. These include studies of glutamic acid antipodes (299), thermomicromethod studies of polymorphic modifications of organic compounds (300), studies of ergot alkaloids (301), ergothionein (302), binary mixtures of some medicinal agents (303), and ternary mixtures (304). With the Thermo-Mikro-Methoden procedures, the eutectic temperatures of 16 hormones were studied, using benzil, acetanilid, phenacetin, benzanilid, salophen, or dicyandiamid. It was noted that 11 of the 16 hormones were polymorphic and that three were present in commercial products in an unstable modifica-

tion (305). Arceneaux has reported that the polymorphic character of trichlorophenoxyacetic acid and related compounds made it difficult to develop a quantitative microscopic fusion method of analysis for commercial products (306). Arceneaux also presented a study of the microthermal analysis of the binary mixture of 1,2,3,4-tetrachlorobenzene-pentachlorobenzene (307).

The great amount of work that McCrone has done with fusion methods can be appreciated by referring to his text (298). His methods have been applied to pharmaceuticals. These include the sterols (308) and esters of 2,4,6-trinitrobenzoic acid (309). The naphthalene molecular addition compounds of these esters melted incongruently, whereas most of the phenanthrene molecular addition compounds melted congruently. Trinitrofluorenone was found to be capable of forming molecular addition compounds with aromatic hydrocarbons (310). From the study, it was concluded that those substituent groups known to release electrons to the benzene ring either by inductive mechanisms or by a resonance mechanism generally led to a molecular addition compound with the reagent. Bulky groups on the benzene ring prevented complex formation. The study was extended to polynuclear aromatics (311), which included the examination of adlehydes, esters, acids, amides, phenols, and aniline derivatives. The reagent is of more limited applicability to the benzene series than to the polynuclear series. The value of trinitrofluorenone was recognized, but a sub-reagent was also desirable for the identification within a series of compounds. Quinones were investigated as possible sub-reagents. Of nine quinones studied, 2,5-biphenyl-1,4-benzoquinone offered the greatest potential (312).

The use of fusion methods was extended to the identification of high explosives (313). For the identification of these compounds, thymol functioned as the solvent for the solute (explosive) crystallizations. Seven different physical characteristics were noted depending on the solubility of the compounds in thymol. It is interesting that McCrone, in this study, stated that fusion methods ensure that a given compound will always show the same morphological characteristics. West and Granatelli utilized 8-quinolinol in developing a fusion method for the microscopic identification of inorganic ions (314).

The fusion method was used in the determination of 2,2-bis-*p*-chlorophenyl-1,1,1-trichloroethane in technical D.D.T. (315). In a complete study of the problem of the impurity in D.D.T., the investigators applied the Arrhenius rate equation to the rate of crystallization of technical D.D.T. with varying percentages of impurity. When the log rate of crystallization was plotted against the reciprocal of the temperature, straight line plots were obtained. Utilizing methods of fusion techniques, the purity of compounds can be estimated by sublimation procedures (316). Petrucci and Weygandt reported that the impurity content could be determined if about 1% or greater. The same method can be used for removing trace impurities from organic solids.

CRYSTALLINE PROPERTIES

Crystallization

Bush reviewed the separation and purification of compounds by crystallization on a small scale (317). Tipson believes that often the best method for separation and purification of organic compounds is by recrystallization (318). He outlined schemes for batchwise, countercurrent fractional recrystallizations, and investigated the distribution of the components in the phases after each recrystallization. He illustrated his theory, scope, and methods with binary and triangular phase diagrams.

Classical multicrystal growths from solution still receives little attention (319). No comprehensive treatment of the batch process has been reported in spite of its wide commercial application. Caldwell reported that crystals of commercially acceptable size could be produced by (*a*) circulation of crystals in the supersaturation zone, (*b*) removal of excess fines, and (*c*) maintaining high magma density (320). He considered these factors in relation to a continuous crystallizer which produces supersaturation by vacuum cooling, rather than by evaporation. The continuous crystallizer, known as the draft tube, which utilizes the above listed factors will produce crystals up to about 16 mesh. Caldwell called attention to the mechanism of crystal formation. He discussed the postulates of Meir, and of Rumford and Bain, concerning nuclei formation. Crystal growth was considered from the viewpoint of bulk diffusion.

Whether crystal growth is bulk diffusion controlled, surface reaction controlled, or interface process controlled has been the subject of study by various workers. Doremus (321) showed that crystal growth rate of ionic salts was controlled by an interface process rather than by bulk diffusion of solute. He proposed an adsorbed layer on the growing crystal, and argued

mation was a fourth-power step. When growth of a constant number of small particles of the same size and concentration was greater than 0.4 mM, the precipitation was diffusion controlled and the kinetics was measured according to the following equation

$$k_0 t = \int_0^a a^{-1/3}(1 - a)^{-1}\, da \qquad \text{(Eq. 22)}$$

When the concentration was less than 0.4 mM, then the kinetics was expressed by

$$k_R t = \int_0^a a^{-2/3}(1 - a)^{-4}\, da \qquad \text{(Eq. 23)}$$

In the above equations a is represented by the degree of reaction (precipitation). Thus, at low concentrations the crystal growth is increased with the fourth power of the concentration. The changes of the order of 0.4 mM can be observed microscopically. When the initial concentration was less than 0.5 mM the crystals were small prisms. When the initial concentration was between 0.5 and 1.5 mM, the crystals were more or less distorted prisms, the corners having grown more than the middle of the faces. When the concentration was greater than 1.5 mM, the corners had grown far more than the other parts of the crystals, giving them appearance of stars. Nielsen stated that the stars were to be expected in the case of diffusional control of growth rate since the concentration then is greatest at the corners. Rectangular growth is obtained when the concentration is approximately the same all over the surface and it cannot be so when the consumption of matter is fast compared with diffusion.

Schlichtkrull has studied the nucleation and growth of insulin crystals (339–344). Nuclei were formed by freeze drying a crystalline mixture containing dissolved insulin. Monodisperse insulin crystal suspension with crystal sizes of 5, 25, and 40 μ were obtained. The rate of nucleation and growth of the crystals as functions of concentration of insulin in crystal mixtures were reported. The rate of growth of pig insulin from a clear solution containing sodium citrate and acetone could be represented by

$$dc/dt = 2.8 \times 10^{-7} \times c\,(95.8 - c)^3 \qquad \text{(Eq. 24)}$$

where C represents the crystalline fraction in per cent of total insulin and is measured in minutes. The linear rate, R_d, of deposition of insulin on crystal faces is

$$R_d = 1.63(C_i - 0.080)^2 \text{ u./min. for saline, beef} \qquad \text{(Eq. 25)}$$

$$R_d = 0.38(C_i - 0.066)^3 \text{ u./min. for citrate, pig} \qquad \text{(Eq. 26)}$$

The concentration, C_i, of dissolved insulin was expressed in mmoles per L. The shape of the crystals could be related to the species. Using ordinary methods, recrystallized pig insulin precipitated as single, sharp, and perfect rhombohedral bodies. Cattle and sheep insulin crystals were often of a twinned appearance. When urea or a halogenide was present in the mother liquid of all three insulins, perfectly shaped rhombohedral crystals were obtained. A patent was issued to Schlichtkrull for obtaining uniform insulin crystals for injection (345). A relationship between the weight of seeds (P), the weight of product desired (I), the size of seed crystals (d_p), and the size of the product crystals desired (d_i) was found to be

$$P = I\left(\frac{d_p{}^3}{d_i{}^3 + d_p{}^3}\right) \qquad \text{(Eq. 27)}$$

Choi and co-workers (346) found that β-lactose could be determined from its rate of crystallization in a saturated lactose solution abundantly seeded with crystalline α-lactose hydrate. In a mixture of β- and α-lactose, the α-lactose could then be determined as the difference between the total lactose and the crystallized β-lactose. The rate of crystallization of β-lactose was represented by

$$k_2 t = \log \frac{c_0 - s_\infty}{c_t - s_\infty} \qquad \text{(Eq. 28)}$$

where the total initial lactose concentration after seeding was C_0, the final solubility S_∞, and the concentration C_t, at any given time t.

Crystal Habit

Crystallographers describe the habit of a crystal as acicular, lamellar, tabular, equant, and columnar (347). The habit of pharmaceutical compounds has been used for purposes of identification. However, this method of identification has definite limitations. It has been stated that "it is virtually impossible to duplicate the crystallization pattern of a single compound on two slides" (348). The angles between any two faces of a crystal will remain the same even if the crystal growth is accelerated or retarded in one direction or another. Should crystal habits be used to identify compounds, the habits from various solvents should be used for differentiation of compounds. The microscopic identification is more reliable the greater the number of crystallizations from various solvents or the greater the number of crystallizations of different derivatives. Identification by this method has been used considerably by chemical toxicologists (349, 350).

In a series of articles Shead discussed the use of profile angles for the identification of compounds.

that fitting a solute unit directly into the crystal lattice from solutions would be difficult. It would be an improbable event for uncharged molecules to collide at a kink in a growth step with just the right orientation to fit into the crystal lattice. For ionic solutes, the difficulties would be even greater because at least two different particles would have to collide simultaneously or alternately in a kink. Furthermore, the ions would have to be desolvated at the same time as they fitted into the lattice. Thus, the concept of an adsorbed layer which permitted desolvation and partial orientation before incorporation into the lattice seemed reasonable.

Bauer (322) considered the general mechanism of crystal growth from a thermodynamic point of view. He stated that the atom layer growth through surface nucleation occurred only for growth on material of the same composition. Growth on a material of different component can occur by original formation of a mono or polymolecular layer without nucleation which then leads to formation of three-dimensional nuclei or through formation of three-dimensional nuclei directly on the foreign substrate. Hoffman (323) called attention to the free energy difference between the rate of nucleation and growth of crystallites in supercooled liquids. The free energy difference was approximated by the expression

$$\Delta F = \Delta H_f \cdot \frac{\Delta T}{T_m} \qquad \text{(Eq. 17)}$$

The equation was derived assuming that ΔH and ΔS in the thermodynamic relation

$$\Delta F = \Delta H - T\Delta S \qquad \text{(Eq. 18)}$$

is constant with temperature, and equal to ΔH_f and $\Delta H_f/T_m$, respectively. In many cases ΔH and ΔS depend on temperature. Hoffman derived a simple expression for the thermodynamic driving force in nucleation and growth processes that takes into account the temperature variation of ΔH and ΔS

$$\Delta F = \left(\Delta H_f \cdot \frac{\Delta T}{T_m}\right)\left(\frac{T}{T_m}\right) \qquad \text{(Eq. 19)}$$

Those interested in the theory of crystal nucleation from vapor, liquid, and solid systems should read Dunning's discussion of the matter as well as the work of Higuchi and O'Konski (324, 325).

Stranski (326), among various workers, developed a classical theory for the growth of crystals. The growth of a crystal depended upon the molecules depositing in layers on a crystal surface. Gibbs (327) stated that a perfect crystal surface could only grow by repeated two-dimensional nucleation of new layers. According to this, a crystal can only grow if the supersaturation amounts to at least 25%. However, Volmer and Schultze succeeded in 1931 in getting iodine crystals to grow further from a 1% supersaturated vapor. This unusually great discrepancy between calculation and experiment was the cause for new investigations. In 1949 Frank succeeded in explaining the discrepancy. He stated that instead of growth occurring by growth in layers, growth occurred in the form of spirals (328, 329). Frank's theory has been tested and positive results have been reported by many investigators. Most recently, Dash and Tweet reviewed the methods of observing dislocations in crystals and discussed the work of Frank and others (330). Studies of growth steps on sucrose crystals (331), paraffin (332), diphenylamine, phenanthrene, and borneol (328) provide interesting reading. The reader should refer also to Buckley's text on "Crystal Growth" (333). Cottrell stated that to understand work-hardening, creep, fatigue, fractures, and recrystallization, one has to study the collective behavior of large, complex assemblies of dislocations, and the theory of this can go forward only when experiments have shown what are the decisive aspects of this behavior in practice (334).

Kinetics

In a study of the kinetics of barium sulfate precipitate formation, Johnson and O'Rourke said the precipitation process is interpreted as being initially controlled by nucleation reaction and finally controlled by growth reaction (335). They reported that the rate of growth of a single crystal could be represented by

$$da/dt = -k(c_0 - c)^{2/3} \cdot a^q \qquad \text{(Eq. 20)}$$

where a = mean ionic activity of Ba^{++} and SO_4^{--}, b = time, and c_0 and c = molar concentration of barium sulfate available at t_0 and t. The kinetic constant and shape factor is incorporated in k. The rate of change of the mean ionic activity as well as the surface of the particle is proportional to some power q. The growth equation for a constant number of particles becomes

$$da/dt = -k_v^{1/3}(c_0 - c)^{2/3}(a - a_s)^q \qquad \text{(Eq. 21)}$$

Further studies on the kinetics of crystal growth in barium sulfate precipitations were reported (336–338). Nielsen found that the work of Johnson and O'Rourke was valid for precipitations of certain electrolytes. In Nielsen's original work he reported that the rate of growth of barium sulfate was controlled by some sort of chemical reaction in which the crystal for-

Using sublimation procedures, he specified the conditions necessary to obtain thin crystal plates of simple goemetric form suited for measurement of profile angles (351–354). McCrone stated, however, that profile angles are not sufficient for identification except in limited cases (355), and suggested that using profile angles along with fusion, optical-crystallographic and polymorphic studies, and rates of crystal growth would be more conclusive. In a series of articles dealing with "organic chemical microscopy," Dunbar and associates developed tests for organic classes of compounds using specific reagents. They prepared derivatives which could be identified microscopically. They reported methods for the identification of amines (356, 357), carboxylic acids, anhydrides and acid chlorides (358, 359), aldehydes and ketones (360), hydroxy compounds (361), amino acids (362), and cations (363). Van der Wegen reported the microchemical identification of some alkaloids, barbiturates, sulfonamides, and new synthetic drugs. Sixty-nine compounds and 29 reagents were tabulated with 42 drawings of crystal forms (364). Sandri has reported that bromoplatinic acid is more sensitive as a reagent for identification of organic bases than is chloroplatinic acid. Bromobismuthic acid was also studied as a reagent. In addition, Sandri used a special potassium bismuth iodide solution for the microchemical detection and differentiation of β-phenylisopropylamine and N-methylphenylisopropylamine (365–367). Berisso utilized potassium iodobismuthate to characterize and differentiate piperazine and α-methylpiperazine. Mixtures could be detected in any proportion (368). Tenger was able to distinguish derivatives of pyridine used in medicine with a modified Dragendorff reagent (369).

The use of chlortetracycline hydrochloride as a reagent for the qualitative detection or quantitative estimation of common cations or anions has been reported (370). The antibiotic did not show any promise. The characterization of certain cations with isoquinoline has been the subject of some reports (371–373). For a detailed study of the identification of elements in the periodic table, the text of Chamot and Mason should be consulted (374).

A differential method using crystalline habits has been reported for the identification of barbiturates and sedatives (84, 86). Other investigators have reported that solvents may greatly affect the habit of the crystal (375, 376). In a study of crystal habit, Stroitelev reported that a less viscous media favored more coarse and more equidimensional crystals for pregmatities and pneumatolytic, in comparison with magmatic and hydrothermal deposits (377). In a study of the crystal habit of salol, Malkin reported the dependence of the form of crystal growth on the rate of growth (378). Transition to an acicular shape occurred when the rate of growth of a crystal of salol increased. When undercooling was decreased, the rate of growth decreased and the growth of acicular crystals was replaced by growth of crystals of regular shape. The various habits of ice crystals obtained at various temperatures under an atmosphere of oxygen, nitrogen, or air with carbon dioxide have been reported (379–381).

Hartman and Perdok (382) related crystal structure and crystal morphology on an energy basis. They concluded that the morphology of a crystal is governed by chains of strong bonds running through the structure. The effective period of such a chain of strong bonds is called a periodic bond chain vector, a P.B.C. vector. The faces of a crystal are classified as (*a*) F-faces or flat faces, each of which is parallel to at least two P.B.C. vectors, (*b*) S-faces or step faces, each of which is parallel to at least one P.B.C. vector, and (*c*) K-faces or kinked faces which are not parallel to any P.B.C. vector. The faces described are listed in order of decreasing importance. In a later paper Hartman reported on his studies of the equilibrium forms of crystals (383). The F-faces were found to be the most important faces. For nonionic crystals it was found that the equilibrium form may also exhibit S and K faces. Hartman stated mathematically under what conditions S and K faces might exist.

Jackson developed an equation which permits a prediction of which faces of any crystals are smooth or rough and hence a prediction of growth morphology (384). The equation permitting this prediction is

$$\alpha = \frac{L_0}{kT_E} \cdot \frac{n_i}{V} \qquad \text{(Eq. 29)}$$

where

$$\frac{L_0}{kT_E} = \frac{\Delta S}{R} \qquad \text{(Eq. 30)}$$

L_0/kT_E represents the entropy change associated with the crystal transformation divided by the gas constant, and n_i/V is the fraction of the total bonding energy of an atom that can be associated with an atomic layer parallel to the face under consideration. When $\alpha < 2$, faces are rough and initiation of new layers is easy. When $\alpha > 2$, faces are smooth and initiation of new layers is difficult and growth on screw locations is important.

Rules have been established for twinning (385). Hartman (386) derived seven possible twin laws. He applied the periodic bond chain theory of crystal morphology to growth twins. He concluded that when twinnng starts on a face, this face must be an F-face or, less probably, an S-face. He stated that the difference of habit between twins and single individuals can be understood from enhanced growth at the twin boundary. This enhancement takes place only where F-faces or eventually S-faces must meet at a reentrant angle of the twin boundary. The twin plane of contact twins should be an F-face. With penetration twins, a certain F-face or S-face can be indicated that acted originally as a composition plane. Hartman stated that the existence of threefold, fourfold, and sixfold twin axes is to be doubted. The elbow twinning that occurs in calcium carbonate has been diagramed by Bragg (387).

The crystal habit has been used to correlate crystal structure with the thixotropic behavior of aliphatic urethanes (388). Hendrickson and Shulman found that all of the long chain aliphatic urethanes showing thixotropic characteristics had fine needle or cluster of needles type structures. The more pronounced the structure of the cluster of needles, the better the thixotropic properties. Compounds showing no thixotropic properties had rod-like crystal structures. One has to examine the photomicrographs to fully appreciate the relationship between the habit and thixotropy.

Shell (389) has emphasized the role that crystal habit often exerts in the development of stable suspensions. Shell stated that in tableting "the mechanical influence of crystal shape . . . is one factor, but there is another, sometimes dominant one, which results from the anisotropy of cohesion and of hardness which is possessed by organic crystals . . . It is significant that this anistropy bears a fixed relation to the fundamental crystallographic directions. Therefore, as crystal habit varies, the dominant faces may vary in their relation to this anisotropy, and it is the influence of the dominant faces which tends to orient the crystals during a packing or compression process. Thus, major habit variations of an active ingredient can influence greatly the ease or the difficulty of making satisfactory compressed tablets." Shell quantitated the crystal habit of various batches utilizing preferred orientation measurements.

Crystal Adsorption

Some of the recent work published concerning the modification of crystal habit by the addition of impurities or poisons should provide a real stimulus. The early studies of crystal habit modifications were summarized by Whetstone (390). Whetstone reported that the crystal habit modification by dyes depended on the anionic and cationic substituent groups and the nature of the substitution. Dyes were adsorbed in a majority of conditions when sulfonic acid groups were present. Several sulfonic acid dyes were used in a study of the alteration of crystal habits of ammonium, sodium, and potassium nitrate, as well as ammonium sulfate. The effects of pH and dye presence on the crystal habit of these salts were correlated. In additional publications, Whetstone illustrated the orientation of the dye molecule in relation to the cations and anions on the crystal face where the adsorption occurred. Pleochroism was utilized in determining the face for dye adsorption (391–393). Michaels, Colville, and Tausch studied the effect of cationic and anionic surface-active agents on the growth of adipic acid crystals (394, 395). The effect of cationic and anionic surface-active agents on the growth of adipic acid crystals was studied. X-ray analysis indicated that the linear 6-carbon dicarboxylic molecules are aligned end-to-end in parallel array in the crystal with their long axes parallel to the 010 face so that the 001 face is made up entirely of carboxyl groups, while the 010 and 110 faces contain both carboxylic and hydrocarbon portions of the molecule. Trimethyldodecylammonium chloride (TMDAC) in concentrations of 0.364 mmole was twice as effective in hindering the growth on the 001 face as on the 110 or 010 face. High concentrations of TMDAC caused the formation of very thin plates or flakes. Concentrations of 50 p.p.m. sodium dodecylbenzenesulfonate (SDBS) were three times more effective in reducing growth rates on the 110 and 010 faces than on the 001 face. Higher concentrations of SDBS caused extreme habit modification, the crystals changing from hexagonal plates to long thin rods or needles. Michaels and Tausch reported that the effect of additive depends strongly on the supersaturation level of adipic acid. At low supersaturation, increasing additive concentration results in drastic reduction or cessation of growth on certain faces, while at high supersaturation levels, the additives have relatively little effect on growth, regardless of concentration. Anionic additives significantly accelerated the growth on the 001 face. Additives increased the supersaturation level at which extensive 3-dimensional nucleation occurred, and resulted in better-formed crystals. Michaels and Tausch interpreted their results in terms of 2-dimensional

nucleation and dislocation theories of crystal growth. Mechanisms for growth and habit modification were developed.

Sears reported that lead chloride in a few p.p.m. altered the growth behavior of potassium chloride (396). Sears attributed this alteration to the inhibition of the rate of growth at unit steps and that the rate of two dimensional nucleation was markedly increased by the presence of the impurity. In a second paper Sears stated that the observed alteration of crystal growth by specific soluble impurities was inexplicable by the Frank theory of crystal growth (397). He proposed that the poison was adsorbed at growth steps to give monostep coverage at each step. The growth rate from a step was then controlled by the equilibrium concentration of single unfilled sites in the line of adsorbed atoms. As the concentration of poison increased the number of open sites along a step decreased, and the rate of step motion then decreased. Sears further proposed that if a poison was adsorbed at a step, it reduced the step energy. Thus a growth poison will reduce the critical free energy for two-dimensional nucleation and increase the nucleation rate. Comer presented evidence that cations including stannic, chromium III, and ferric were adsorbed on the surface of primary ammonium phosphate and primary ammonium arsenate (398). The tapering of the crystals in the presence of the foreign cations was explained by the presence of the foreign cation at the intersection of the 100 and 101 plane and the 010 and 011 plane. This inhibited the growth and caused successive layers to deposit at a slight distance in from the intersection of these planes. McCartney and Alexander reported that the rate of nucleation and crystal growth of calcium sulfate dihydrate was most strongly reduced by colloidal materials having regularly ionized carboxyl groups on a chain structure (399). The anionic polymers were most strongly adsorbed on the 111 faces of the crystals.

Polymorphism and Lattice Energy

In 1821 Mitscherlich discovered polymorphism with his work on the dimorphism of calcium carbonate. Since that time a considerable amount has been reported in the literature. In 1958 Eitel reviewed the importance of polymorphism in geological problems; his review included 700 references (400). O'Conner's chapter on the polymorphism of fatty acids represents an excellent review of the massive literature on the subject (62). Additional sources concerning polymorphism include Hartshorne and Stuart (401), Kofler and Kofler (296), McCrone (298), and Jelley (192).

The importance of polymorphism has increased in recent years in pharmaceutical research for several reasons. Analytical errors may develop when analytical methods utilizing physical properties change with a change in crystal structure (306). Polymorphs of a drug differ in total solubility in a given solvent (402). The absorption rate can vary with different polymorphs of a given drug (403). Polymorphism can create problems in terms of crystal growth in suspensions (404). Polymorphic forms can create problems involving differences in filterability (405). The thermal stability can vary with different polymorphs of a drug (298). The success of a new product is seldom independent of its physical form (406).

Polymorphism or transitions from one polymorph to another can be detected by various methods. Observation of transitions with temperature can be easily detected using the Kofler melting point block. Dilatometry has been used to study polymorphism of fats and other compounds (407, 408). Cini and co-workers believe that changes in magnetic anisotropy provide a valuable tool in the study of polymorphism (409). Optical crystallography has been a most valuable tool (253), as has X-ray crystallography (410). Electron microscopy has been utilized for such identifications (411). Infrared spectroscopy has been used with success (412). Surface tension measurements have been utilized in determining the transition temperatures (413, 414).

Various methods can be used for the preparation of various polymorphs of a given compound. Pressure has been used (415, 416). This reviewer has not read any articles dealing with the polymorphism of drugs as affected by pressures used in tablet compression. Temperature changes have been widely used for the preparation of polymorphs and phase diagrams for the temperature variable have been discussed (192, 296, 298, 401). Impurities have been known to cause polymorphism (191), as well as mode of crystallization (191). Solvents have been used for the preparation of polymorphs.

Some fundamental studies of polymorphism are lacking. However, von Sydow (191) attempted to study the dipole effect of the solvent on the polymorphic form of fatty acids isolated, and reported that the study was difficult because the rate of crystallization was not controlled. A second way that the solvent could affect the crystallization was related to the vapor pressure

of the solvent. Two polymorphs of chloracetamide have been isolated using solvents (417). The difference between the cell dimensions was found to be only in the value of the obtuse angle in the unit cell. The alpha form was obtained from nonpolar solutions. These crystals were hard to dissolve in benzene or in carbon tetrachloride. Katayama suggested that these molecules become stable in these solutions by making dimers by hydrogen bonds, and the dimers thus formed come into the crystals having the alpha configuration. These crystals are quite soluble in water or ethyl alcohol and the molecules make hydrogen bonds with the solvent molecules. Katayama concluded that "this will be the reason why the crystals obtained from these solutions have a different structure." In a study of the polymorphism of 17-β estradiol, Smakula, *et al.*, stated that the ability of molecules of a compound to associate in alternate modes of hydrogen bonding of comparable stability can be expected to be a major cause of polymorphism, thereby causing differences in the solid-state spectra of polymorphic substances (418). W. Higuchi, *et al.*, in a study of the polymorphism of methylprednisolone, reported that the lack of structural information on the methylprednisolone system precluded a single interpretation of the entropy of transition. But they suggested that the entropy difference was a result of greater localization of the functional groups in the side chain in form I resulting either from intermolecular or intramolecular interactions (419).

Because of the similarity in structures, the polymorphism of hydrocortisone terbutylacetate and prednisolone terbutylacetate was studied (420). Four polymorphs of each system were isolated, but the isolation methods were not similar.

The transition rates of some polymorphic systems have been studied. Hartshorne and Thackray have studied the α-and β-sulfur systems (421). The activation energy for the transfer of molecules from the beta to the alpha form corresponded closely to the heat of sublimation of the beta form. Thus the molecules in the transition layer at the interface were energetically equivalent to the molecules in the vapor state. Johansson (422) suggested that should the formation of nuclei be a homogeneous process and that all crystals be of the same size, the probability of transition should be the same for each crystal. The course of the transition could be described as

$$-dN/dt = KN \qquad \text{(Eq. 31)}$$

$$N = N_0 e^{-kt} \qquad \text{(Eq. 32)}$$

where N is the number of nontransformed crystals and N_0 is the number of crystals when time is zero. However, when log N was plotted against time, a straight line was not obtained. Thus, the reaction must be heterogeneous and described by

$$N = N_1 e^{-k_1 t} + N_2 e^{-k_2 t} + \ldots \qquad \text{(Eq. 33)}$$

The heat of activation could not be determined for the various nuclei so no conclusions could be made concerning whether or not the number of nuclei was proportional to the surface or the volume of the crystals. Johansson examined the kinetics of the polymorphic transformation since such an investigation might elucidate the influence of a lattice transition on the velocity of reactions in the solid state. In a study of the growth of crystals in the alpha to beta transition in paradichlorobenzene, Bykhovskii, *et al.*, found that the linear growth rate was a function of temperature (423). The temperature dependence of the rate was not in agreement with Hartshorne's supposition that the rate of displacement of the boundary between the phases was determined solely by the difference between the rates of evaporation. Bykhovskii's conclusion was that the material was transferred directly in the solid state as a result of thermal agitation. In the study of the transition rates the results agreed best with the supposition that the alpha to beta, or vice versa, transition in paradichlorobenzene involved a dislocation mechanism in growth.

The rate constant for the reaction of phenylhydrazine with the stable and unstable modification of *o*-methoxybenzaldehyde was determined by Urazovskii (424). Using the metastable form of the aldehyde the rate constant was found to be 4.15 at 3.5°, and persisted at 5, 15, and 25°. The stable form has a much higher rate constant than that of the metastable form. In aqueous dioxane the difference was greater than in aqueous ethanol. The stable form was apparently a hydrogen bonded dimer, whereas the metastable form was chelated by a hydrogen bond. The heat of activation for the stable form was 11,400 cal. per mole and 11,200 cal./mole for the metastable form.

Structural analysis has been valuable in the study of polymorphism. The crystal structure and polymorphism of N-methylacetamide has been reported by Katz and Post (425). The low temperature form changed to the high temperature phase at 10°. Below the transition temperature the unit cell was found to be orthorhombic with 4 molecules per cell. Above the transition temperature, the orthorhombic unit cell contained 2 molecules per unit cell. Along with

this transition there was a reduction in cell size which was brought about by the existence of molecular orientational disorder. The polymorphism of glycine has been studied in detail (183, 184, 426). Three forms, alpha, beta, and gamma, have been isolated. The beta form is unstable. The molecular volume of the three forms was the same within one cubic Å. β-Glycine consisted of hydrogen bonded molecular layers extending parallel to the 010 plane. The layers have the same configuration of molecules as the alpha form. Single layers in the beta form were connected geometrically by the twofold screw axis and held together by hydrogen bonds extending in the *b* direction. In α-glycine the nitrogen atom was buried within the double layer in such a way that very close contact of these two layers occurred. Where molecular layers were discovered in the β-glycine parallel to the *c* axis, molecular double layers held together by hydrogen bonds were found parallel to the *c* axis in α-glycine. γ-Glycine, which showed a marked piezoelectric property along the *c* axis, was found to have a crystal structure nearly the same as that of α-glycine. The bond distances and bond angles were compared with γ- and α-glycine and with α- and β-glycine. The crystal structures of the three forms of the even-numbered fatty acids, *A*, *B*, and *C*, and the four polymorphs of the odd-numbered fatty acids, namely *A'*, *B'*, *C'*, and *D'* are illustrated and discussed by von Sydow (191, 427).

The crystal structures for the polymorphs of acridine have been studied. Acridine was reported to have five distinct polymorphic forms (428). It was found that acridine I was a hydrate. Acridine II and III appeared to have nearly the same free energies at room temperature since neither showed any great tendency to transform to the other and both were produced by the decomposition of I. At higher temperatures II was the stable form. The sizes of the unit cells, lattice energies, and volumes per molecule were almost equal (172–174). A comparison of the packing of anthracene and acridine III was shown. The acridine II consisted of two molecules in the asymmetric unit cell and exhibited significant departure from planarity. This was also evidenced from the lack of fluorescence of the crystal. Birks and Cameron reported that fluorescence in aromatic compounds is associated with planarity (429). The fluorescence was determined by the presence and configuration of the π electrons in the molecule. Phillips, *et al.*, reported that the apparent molecular thermal motions in I, II, and III were of the same type but that the translational components of the mean square amplitudes of vibration appeared to be higher in the acridines than in the anthracenes. This result may explain the lower melting point of the acridines than anthracene. Phenazine has been found to be dimorphic (430, 431). Indigo has been found to be dimorphic. The value of $a \cdot \sin \beta$ was found to be the same for the two forms. However, the change of one form to the new form occurred by a slip of about $c/4$ of the molecular plates parallel to the *YOZ* plane (432).

One of the most difficult polymorphic problems to solve was that of cortisone acetate. Several patents have been issued (433–440). The polymorphs of cortisone acetate were detected by infrared spectra (441, 442). Most recently, Callow and Kennard correlated the previous work (443). There is evidence of six crystalline modifications or solvates of cortisone acetate. Five forms of cortisone acetate and methods of their isolation along with photomicrographs were presented by Callow and Kennard. They reported that forms II, III, IV, and V as intact crystals are stable for some time in the presence of water, but when shaken or ground, transformation to form I takes place rapidly. Crystals of form II were apparently unchanged in water after 126 hours, whereas in a shaking machine a change was seen after 20 hours and was complete after 60 hours. If the crystals were ground, transformation could take place in 4 hours, but probably because of caking of suspensions, complete transformation still took about 56 hours. Form IV was found to be a dihydrate which was found to be unstable in water at room temperature. This reviewer, in a study of the polymorphism of cortisone acetate, noted that crystals from aqueous alcohol, presumably form IV, gradually changed to another form when stored at room temperature free from solvent. This change was followed easily by noting the change in the refractive indexes of the crystals.

Polymorphism has been recorded for many steroids and natural and synthetic drugs. It would be impossible to list all of the forms of the various drugs that have been reported in the literature. Though temperature studies have been reported, infrared patterns interpreted, speculations on hydrogen bonding made, little or nothing can be said of the exact molecular orientations. Much work must be done, and perhaps it is up to the X-ray crystallographers to move in.

Polymorphism has been investigated from a thermodynamic point of view. Staveley discussed, in length, the transitions in solids and liquids (444). Hurst obtained a second-order differential equation in the variable p and T which is valid for all phase equilibria of the first and second orders (445). A first-order phase transition is accompanied by the absorption or evolution of latent heat. Second-order transitions differ from first-order transitions in that there is no evolution or absorption of latent heat. Hurst says that a second-order transition is to be regarded as the limiting case of a first-order transition with infinitesimally small ΔV. The heat, entropy, and temperature of transition have been calculated for the forms I and II of methylprednisolone (419). The escaping tendency, or fugacity, for the two polymorphs was determined by solubility studies. It was found that the transition temperature was 118°, that the $\Delta H^{\circ}_{I,II}$ was 1600 cal. per mole, and that the entropy change at the transition point was 4.1 e.u. Exploratory studies involving cloud point determinations indicated that much more energetic crystal forms were probable.

Frederick (404) reported that one of the most important elements of polymorphs is that the different forms of the same drug differ in their solubility properties. Wagner (446) pointed out the significance of differences in dissolution rate *in vivo* of drugs (of different brands of dosage forms of the same drug). The dissolution rate of a compound is proportional to the solubility of the drug in the solvent (447). From these relationships it can be concluded that one polymorphic form may have a greater activity than a second polymorphic form. Higuchi, *et al.* (419), stated that the activity of the more energetic form of methylprednisolone was found to be of the order of 80% greater than that of the more stable form. In an *in vivo* study of the forms I and II of methylprednisolone, Ballard and Nelson (403) found that the mean absorption rate in mg. per hr. per cm.^{2} was approximately 1.7 times as great with form II than with form I. This study was performed by preparing pellets of the drug for implantation. Rats were used in the investigation. In discussing the physical chemical analysis of percutaneous absorption, Higuchi stated that substances showing lower melting points generally permitted higher concentration in solution and would thus tend to give faster penetrating systems. He stated that it would be difficult to produce rapid absorption of high melting point compounds (448). Higuchi also pointed out that different crystalline modifications may exist having different free energies, thus different thermodynamic activities at room temperature. As to the choice of polymorphs of a given drug, it may be pointed out that in 1952 Dale received a patent for the development of a polymorph of riboflavin which possessed much greater solubility and therefore a more useful form of the vitamin (402).

One can conclude then that the lattice forces are important in terms of the availability of the drug for utilization. Pirsch has contributed to the question of the lattice forces of organic molecules (449). In recognition that the form of the spatial filling of organic molecules is of decisive influence on the magnitude of the molar heat of fusion, Pirsch arranged organic molecules into three large spatial types, according to the magnitude of their molar heat of fusion. These three spatial groups were classified as (*a*) chain molecules, (*b*) disk molecules, and (*c*) spherical molecules.

Organic compounds of the spherical structures show small fusion entropy values and low molar heats of fusion. As a rule, these types of compounds demonstrate no polymorphic changes near the melting point. Closely above the melting point, liquid crystals should persist. The molar heat of fusion is greater for the disk and chain compounds, with the latter showing the highest values. A relationship was presented characterizing the linear dependence of the molar heat of fusion on the position of the melting point

$$\Delta_E H = k(T_e - a) \qquad \text{(Eq. 34)}$$

in which k and a are characteristic constants for each spatial form type. Just as a higher energetic form would be desirable for drug availability *in vivo*, a higher energetic form may not be the most suitable form for drug stability. Higuchi and Guillory determined the stabilities of derivatives of vitamin A (450). The investigators found that the relative rates of degradation of series of similar solid derivatives measured at any temperature were such that the logarithm of the rates was a linear function of the reciprocal of their melting points. Thus a strong lattice offers a stabilizing effect.

An additional consideration in drug availability from the crystalline state would be solvates. Rose, *et al.*, have reported that *trans*-diethylstilbestrol forms solvates when crystallized from chloroform, benzene, ether, 2-propanol, methanol, and acetic acid (214). Rose also reported that erythromycin formed an acetone and chloroform solvate which readily lost solvent

on exposure to air and converted to a powder (279). Gramicidin forms a solvate with acetone which is apparently lost on drying. Different forms were obtained from various solvents (451). Ballard and Nelson (403) reported that anomolous results were obtained when anhydrous tetracycline pellets were implanted *in vivo* (rats). They suggested that the anhydrous tetracycline at body temperature was converted to the trihydrate. Higuchi and Shefter (452) recognized that many drugs exhibit a strong tendency to form discrete hydrate structures, but that much less was known concerning the formation of solvates with organic solvents. These investigators showed that the normal amyl alcohol solvate of fludrocortisone acetate had at least five times the solubility of the nonsolvated species, while there was a twofold increase with the ethyl acetate solvate. The anhydrous forms of caffeine, theophylline, glutethimide, and cholesterol showed a greater dissolution rate than did the corresponding hydrates.

A different approach to drug availability was reported by Sekiguchi and Obi (453). These investigators compared the absorption of sulfathiazole to an eutectic mixture of sulfathiazole. Eutectics with sulfathiazole were prepared using ascorbic acid, acetamide, nicotinamide, nicotinic acid, succinimide, or urea. The urea eutectic was studied *in vivo*. Where sulfathiazole had a melting point of about 200°, the eutectic mixture of 52% sulfathiazole and 48% urea by weight melted at 112°. It was reported that the solubility of sulfathiazole decreased slightly with a continued increase by weight of urea. In a study of the report Nelson (454) stated that the 8-hour cumulative urinary excretion of the eutectic was 50%, while the 8-hour cumulative urinary excretion of the normal sulfathiazole was 39%. In Nelson's studies he reported that using finely powdered sulfathiazole the 8-hour cumulative urinary excretion was 75%.

It is evident that similar studies will follow. A fruitful area should be complexation. Drugs forming clathrates, channel lattice type complexes, as well as the possible complexation of nonpolar aromatics with heterocycles could be investigated. It would be well at this point for the investigator to review the fusion studies of the Kofler group and the McCrone group.

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Research Articles

Infrared Analysis of Pharmaceuticals III

Identification and Determination of Adrenocortical Steroids, Barbiturates, and Sulfonamides from Paper Chromatograms

By ALMA L. HAYDEN

The adrenocortical steroids: cortisone, hydrocortisone, and 17-hydroxy-11-desoxycorticosterone, have been identified and estimated from paper chromatograms in amounts between 50 mcg. and 1.5 mg. Recoveries of 90 to 105% of these steroids were obtained on the basis of infrared spectrophotometric determinations. Analyses were made of commercial tablets which contained (*a*) hydrocortisone and 17-hydroxy-11-desoxycorticosterone, or (*b*) the sodium salts of phenobarbital, butabarbital, and pentobarbital. The results agreed within 3.8% with those published earlier (9), or with the declared amounts. In addition, the identification and estimation of sulfanilamide in a sulfacetamide powder were achieved by these methods.

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In addition to other applications, paper chromatography and infrared spectroscopy have been combined in the investigation of steroids in human placenta (1). Recently, the use of these

Received September 5, 1961, from the Department of Health, Education, and Welfare, Food and Drug Administration, Division of Pharmaceutical Chemistry, Washington 25, D. C.
Accepted for publication October 6, 1961.
The author acknowledges the advice of Mr. Jonas Carol and Miss Frieda M. Kunze during this investigation. The die for the pressed paper peripheries was constructed by Mr. Willie J. Banks.

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In addition to other applications, paper chromatography and infrared spectroscopy have been combined in the investigation of steroids in human placenta (1). Recently, the use of these

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methods in studies of triamcinolone and related steroids (2), and of caffeine, antipyrine, and phenacetin from human tissue (3) has been reported. Techniques have been published (4–6) whereby the spectra of 100 mcg. and less of some pure crystalline compounds in potassium bromide have been obtained. However, impurities in filter paper and solvents have made the analyses of microgram quantities from paper chromatograms extremely difficult. The instability of some compounds and solvents to evaporation techniques has magnified this problem.

In the present study, mixtures of cortisone and hydrocortisone, in amounts between 50 mcg. and 1.5 mg., were separated on washed Whatman No. 1 filter paper with a chloroform-formamide solvent system (7). After extraction from the chromatograms, the steroids were identified by their infrared spectra in potassium bromide plates and disks. The spectra of samples in amounts less than 100 mcg. were obtained using corresponding blanks in the reference beam and the Perkin-Elmer ordinate scale expander (8). The spectra of samples in amounts greater than 180 mcg. were obtained without compensation or spectrum expansion.

The combined method was applied to commercial tablets which contained hydrocortisone and 17-hydroxy-11-desoxycorticosterone, or pentaerythritol tetranitrate and the sodium salts of phenobarbital, butabarbital, and pentobarbital. The results of the analyses agreed within 3.8% with those reported previously (9), or with the declared amounts. In another application, a commercial sample of sulfacetamide was found to contain about 17% sulfanilamide.

EXPERIMENTAL

Preliminary Infrared Study of the Corticosteroids.—A preliminary study was made to determine the practical amounts of pure cortisone, hydrocortisone, and 17-hydroxy-11-desoxycorticosterone which could be identified by infrared spectroscopy. Dried residues of the steroids, from chloroform and from methanol solutions (0.50 mg. or 1.00 mg. per ml.), in amounts of 50 mcg. to 1.0 mg., were investigated. Amounts of 250 mcg. and above were hand-ground with 200.0 mg. of dry potassium bromide (Harshaw, infrared quality, 200/325 mesh) for exactly 2 minutes. Amounts of 50 to 250 mcg. were hand-ground with 40.0 mg. potassium bromide. The mixtures were dried at 105° for 30 minutes and cooled to room temperature.

A Beckman evacuable die, 0.5 inch in diameter, was used to press the mixtures. A force of 20,000 or 25,000 pounds was applied during evacuation. The 200-mg. loads were pressed into disks 12.7 mm. in diameter. Portions of the 40-mg. loads were pressed into 3 × 10 mm. plates in pressed paper or potassium bromide peripheries (10–12) 12.7 mm. in diameter. Approximately 30 to 35 mg. was needed to fill the 3 × 10 mm. window. The disks and plates were heated at 105° for 15 minutes, and were cooled to room temperature in a desiccator.

The infrared spectra were obtained on a Perkin-Elmer model 21 double-beam spectrophotometer which was equipped with sodium chloride optics, a microcell adapter, and an ordinate scale expander (8). The sample plates and disks were inserted in the sample beam with a holder which fitted the microcell adapter. An optical wedge was placed in the reference beam to compensate for opacity of the pressed paper peripheries. For amounts of 180 mcg. and above of the steroids, spectra of the region between 2 and 15 μ were obtained using the normal (1×) qualitative instrument settings. The spectra of amounts less than 180 mcg. were weak (less than 20% absorption) and poorly defined under the 1× conditions. Therefore, the ordinate scale expander (8) was employed to magnify electrically the pen movement, relative to the optical wedge movement, to five times (5×) its normal (1×) movement. The resulting spectra were similar to, but less defined than, those of amounts greater than 180 mcg.

Spectra of pure compounds which absorb less than 10% or 5% of the incident radiation may be expanded 10 and 20 times, respectively. However, interference from trace impurities in paper chromatogram extracts and poorly resolved spectra limit the usefulness of these expansions.

Paper Chromatography of the Corticosteroids.—Just prior to use, Whatman No. 1 sheets (8 × 8 in.) were eluted, continuously, with methanol for at least 4 hours in an ascending chromatograph tank (13). The sheets were air-dried for at least 30 minutes and were viewed in ultraviolet light (2453Å.). The strongly fluorescent solvent-front area (about 1 cm. deep) along the top edge of the sheet was discarded. The starting line was ruled on the side of each sheet 3 cm. from the edge opposite the solvent-front area. The sheets were used within 24 hours after washing.

About 200 ml. of chloroform (U.S.P. or A.C.S. specifications) was equilibrated with 50 ml. of redistilled formamide for at least 30 minutes. Chromatography tanks were lined with blotter paper and were equilibrated with the chloroform phase for 30 minutes prior to chromatography (13). A solution of the equilibrated formamide in methanol (30:70) was used to impregnate the washed sheets. Excess immobile phase was removed by placing the impregnated sheets between layers of paper towels.

Aliquots of methanolic solutions of cortisone and hydrocortisone (about 1.0 mg./ml.) were applied in mixtures along the starting line of the impregnated sheets. Aliquots containing 50 to 100 mcg. of the steroids were applied, with intermittent air-drying, in spots 1 cm. in diameter. Amounts of 180 mcg. to 1.0 mg. were applied in streaks, 1 cm. × 3 cm. or 1 cm. × 14 cm., in the same manner. Amounts between 100 and 180 mcg. were not studied. After application of the samples, portions of the formamide-methanol solution (0.01 or 0.20 ml.) were added to the spots or streaks to reduce sample adsorption at the starting line (14). The sheets were subjected to ascending chromatography for

about 1 hour. During this time, the solvent traveled approximately 18 cm.

The chromatograms were dried, partially, at room temperature for 30 minutes. The steroid zones were located by their ultraviolet absorbance, using a phosphorescent screen (15), and were outlined on tracing paper. The corresponding zones on the partially dried chromatograms were cut into small rectangular pieces and were suspended in 4.0 ml. of dilute hydrochloric acid (about 0.0025 *N*) in separators. The suspensions were extracted with four 4.0-ml. portions of chloroform, allowing at least 1 minute for each extraction. The extracts of each suspension were washed, successively, with the same 3.0-ml. portion of water, and were filtered through chloroform-washed glass wool into a 50-mm. mullite mortar. The 4.0-ml. extracts of each zone were concentrated, in succession, under nitrogen at about 35°. The extracts were evaporated to dryness only after addition of the final portion of chloroform. The residues were dried in vacuum over phosphorus pentoxide for 30 minutes.

Infrared Spectrophotometry of the Extract Residues.—The dried residues were made into plates or disks as described for the direct standards. For amounts of 180 mcg. and more, the normal (1×) spectra were obtained without blank compensation or ordinate expansion. The spectra of the extracted steroids, in amounts of 100 mcg. and less, were weak and poorly defined, and were affected by small amounts of impurities from the paper and solvents. As a result, compensation with a paper-blank extract and 5 × expansion of the ordinate scale were required to obtain identifiable spectra.

Preliminary studies revealed that compensated spectra may be obtained in two ways. In the instrument used, the microcell-sample position is recessed from the macrocell-sample and reference positions. As a result, the reference beam is larger and less intense per unit area at the sampling position than is the sample beam at the microcell-sample position. A standard disk or plate placed in the latter position absorbs about 1.1 times more strongly than when it is placed in the macro-sample position. Therefore, for compensation, a disk of given effective concentration (concentration × thickness) in the micro-sample position requires a disk 1.1 times as concentrated in the reference position (method I). In method II, since the beams at the macro-sample and reference positions are equivalent, a disk in the macro-sample position is compensated by one of the same effective concentration in the reference position (method II). The holders for the macro-sample and reference positions were rectangular metal plates, 74 × 50 mm., with a central 30 × 7 mm. aperture. Tension clips were soldered on each side of the aperture to position the disks or plates.

Therefore, for compensation, blank paper zones, taken from the same chromatogram and corresponding in weight to the 50 to 100-mcg. steroid zones, were extracted in the manner described above for the steroid zones. The dried residues were ground with 36 mg. (method I) or 40 mg. (method II) of potassium bromide. The mixtures were treated in the same manner as was described for the direct steroids.

In method I, uncompensated scans of the 2 to 15 μ region were made of the sample and of the blank plates in the microcell-sample position under 1 × conditions. At the wavelength of maximum transmission (usually near 5 μ) with the sample in the beam, the blank plate was adjusted in the reference beam to give approximately 95% transmission. The compensated spectrum was obtained between 2 and 15 μ using 1× instrument settings. At the wavelength of maximum transmission, the instrument conditions were changed to those of 5× expansion. The pen was adjusted to 95% transmission with the pen position control. The instrument was reversed to 2 μ without further change. A slow scan of the 2 to 15 μ region was made. In method II, uncompensated scans of the 2 to 15 μ region were made of the sample and of the blank plates in the macro-sample position under 1× conditions. All other operations were identical to those used in method I.

The spectra of the samples were compared with those of disks and plates of the direct standards. The estimated recoveries of cortisone and hydrocortisone were calculated from the normal 1× absorbance at 5.82 μ or 6.02 μ (9) by comparison with the direct standards. The absorptivity coefficients for the direct standards, K_s, were calculated from the equation

$$K_s = (A_s \times H_s)/(C_s \times L_s)$$

In this equation, A_s is the baseline absorbance and H_s is the band width at one-half the height of baseline absorbance of the chosen band, C_s is the ratio of the weight of the standard to the weight of potassium bromide, and L_s is the average thickness of the disk or plate area exposed to the sample beam. The recoveries of the chromatographed steroids were calculated by inserting the appropriate absorptivity coefficient in the following equation

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{100}{W} = \text{per cent recovery}$$

A_u and H_u represent the baseline absorbance and half-band width of the sample band, L_u is the average thickness of the exposed sample disk or plate, M is the weight of potassium bromide, and W is the weight of the applied sample.

Analyses of Commercial Products

Hydrocortisone Tablet.—A commercial tablet, declared to contain 20 mg. of hydrocortisone, was suspended in 10 ml. of 0.01 *N* hydrochloric acid. The suspension was extracted with four 25-ml. portions of chloroform. Each chloroform extract was washed with the same 5-ml. portion of 5% sodium carbonate and, finally, with 5 ml. of water. The chloroform was evaporated at about 35° under a stream of nitrogen. The residue was dissolved in absolute methanol and was diluted to 25 ml. in a volumetric flask. A 1.0-ml. aliquot was applied in a streak (1 × 14 cm.) to the starting line of a washed, impregnated sheet of Whatman No. 1 filter paper (8 × 8 inches). The sheet was subjected to ascending chromatography for 50 minutes, and the steroids were detected, extracted, and incorporated into disks (200 mg., 12.7 mm. in diameter) as described earlier.

The spectra were compared, under 1× conditions

between 2 and 15 μ, with those of standard hydrocortisone and 17-hydroxy-11-desoxycorticosterone. Quantitative baseline absorbance and half-band width measurements were made at 5.82 μ for hydrocortisone and at 5.99 μ for 17-hydroxy-11-desoxycorticosterone. The absorptivity coefficient, K_s, for the appropriate standard was inserted in the following equation to obtain the amount of steroid per tablet

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} = \text{mg. per tablet}$$

The terms A_u, H_u, L_u, K_s, and M were described earlier. V_a represents the aliquot of the total volume, V_t, taken for analysis.

Tablets of "Triple-barb" (total, 8.0 mg.) and Pentaerythritol Tetranitrate (10.0 mg.).—A sample of powdered tablet mixture containing 8.0 mg. of "Triple-barb" (sodium salts of phenobarbital, butabarbital, and pentobarbital, in equal amounts) was suspended in 5.0 ml. of water in a separator, and 5.0 ml. of 10% sulfuric acid was added. The active ingredients were extracted with chloroform, and the barbiturates were trapped on a Celite-potassium phosphate column as described by Carol (16). The amount of pentaerythritol tetranitrate in a 25.0-ml. aliquot of the chloroform effluent was determined as described (16).

The barbiturates were eluted from the column with chloroform-acetic acid (150 + 15 ml.) and the solvents were evaporated at about 55° under a stream of air. The barbiturate residue was dissolved by warming in three portions of acetone-hydrochloric acid (15 + 0.5 ml.). The solutions were filtered through glass wool, and the combined filtrates were concentrated to about 2 ml. as described above. The concentrate was diluted to 5.0 ml. with acetone.

Immediately, the barbiturates were separated by a modification of the Sabatino procedure (17). A 0.50-ml. aliquot of the barbiturate solution was applied in a streak to washed filter paper. The filter paper was mist-sprayed with 0.5 M sodium carbonate and was subjected to continuous ascending chromatography (13) for 4 hours with ethylene chloride as the mobile solvent.

The chromatogram was air-dried, and the three ultraviolet absorbing zones were suspended in 5.0-ml. portions of 5% hydrochloric acid. The suspensions were extracted and the extracts were treated as described for the steroids. The extract residue of each barbiturate was dissolved in 0.50 ml. chloroform; 3.0 ml. n-heptane was added, and the solvents were evaporated under nitrogen at about 35°. Crystalline samples of the standards (about 250.0 mcg.) were treated in the same manner. Potassium bromide disks (200 mg., 12.7 mm. in diameter) were prepared of the solid residues. The disks of phenobarbital and butabarbital were heated at 105° for 30 minutes. Because of difficulty in controlling the polymorphic forms, the disks of pentobarbital were heated above the melting point of pentobarbital at 143° for 30 minutes. The disks were cooled to room temperature, and the uncompensated quantitative spectra were obtained between 5.3 and 6.6 μ using normal 1× instrument conditions.

From the baseline absorbances A_u of the bands near 5.65 and 5.85 μ, and the average thicknesses L_u of the disks, the amounts of the barbiturate sodium salts per tablet were calculated from the following equations

$$A_s/C_s \times L_s = K_s$$

$$\frac{A_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} \times \frac{T}{W} \times Z = \text{mg./tablet}$$

The terms A_u, L_u, M, V_t, and V_a were described earlier. T and W represent the average weight per tablet and the sample weight, respectively. Z is the ratio of the molecular weight of the sodium salt to that of the barbituric acid.

The identities of the chromatographed barbiturates were proved by comparing their spectra (1×) in the 2 to 15 μ region with those of the direct standards.

Mixture of Sulfacetamide and Sulfanilamide.—A 100.0-mg. sample of the commercial powder was dissolved in 1.0 ml. of ammonium hydroxide and the solution was diluted to 10.0 ml. with water. A methanol-washed filter paper sheet was impregnated with formamide-acetone (30:70) as described by Maienthal and co-workers (18). A 0.100-ml. aliquot of the sample solution was applied in a streak (1 × 14 cm.) as described previously. The filter paper was subjected to continuous ascending chromatography for 3 hours with chloroform-tertiary butyl alcohol (100 + 6 ml.) as the mobile solvent.

The sheet was air-dried in the dark for about 1 hour and was viewed, briefly, under ultraviolet light. The ultraviolet-absorbing sample zones were outlined, cut into small rectangles, and extracted by warming with four 10.0-ml. portions of acetone for 15 minutes. Each extract was filtered through glass wool, the filtrates were evaporated to near dryness, and the concentrate was transferred to a 50-mm. mortar with several small portions of acetone. The sample solutions and 2.0-ml. aliquots of standard sulfacetamide and of sulfanilamide in acetone (0.200 mg./ml.) were evaporated to dryness as described previously. The residues were dried at 80° in a vacuum oven for 1 hour. The cooled residues were triturated with 2 ml. of heptane for about 10 minutes and the solvent was evaporated at 35° under nitrogen.

Potassium bromide disks (200.0 mg., 12.7 mm. in diameter) were prepared of the sulfacetamide standard, and sample residues, and of the sulfanilamide standard. A potassium bromide rectangle (3 × 10 mm.) in pressed paper periphery was prepared of the sulfanilamide paper chromatography residue. Quantitative spectra were obtained of the heated standard and sample disks and plate between 8.0 and 9.75 μ. Measurements were made of the baseline absorbance and the band widths at two-thirds the height of absorbance of the 9.16 μ band. The per cent of each compound in the commercial mixture was calculated using the equation

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} \times \frac{100}{W} = \text{per cent recovery}$$

The identities of the extract residues were proved by comparison of the spectra (1×) of their disks or plates in the 2–15 μ region with those of the direct standards.

RESULTS AND DISCUSSION

Table I shows that cortisone and hydrocortisone are well separated even in 1:8 and 11:1 mixtures. Except for slightly lower values for the 50-mcg. zones, the mobilities were reproducible.

In Fig. 1 are spectra of chromatographed cortisone (II, 180 mcg.) and hydrocortisone (IV, 1.46 mg.) obtained under normal 1× conditions. The uncompensated spectra are identical to those of the corresponding direct standards (I, 180 mcg.; and III, 470 mcg.) and to published spectra (12). Figure 2 gives expanded (5×) spectra of chromatographed cortisone (I, 50 mcg.) and hydrocortisone (II, 100 mcg.) compensated with corresponding blanks. In general, the expanded spectra are similar to but less resolved than those obtained under normal operating conditions. The background absorbance in the C—H stretching and bending regions is due to uncompensated impurities from the paper and solvent.

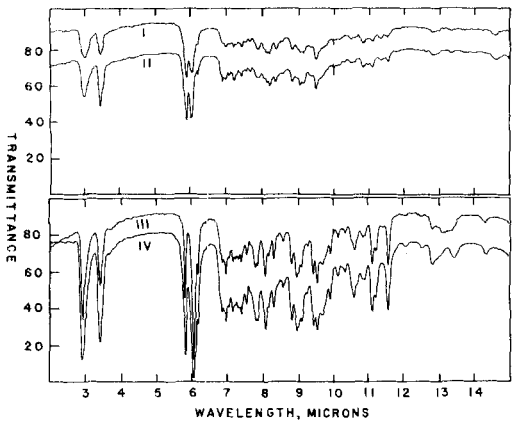


Fig. 1.—Infrared spectra of chromatographed cortisone (II) and hydrocortisone (IV) and the corresponding direct standards (I and III).

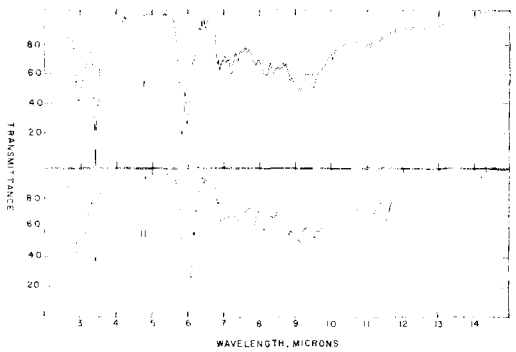


Fig. 2.—Expanded (5×) infrared spectra of chromatographed cortisone (I, 50 mcg.) and hydrocortisone (II, 100 mcg.)

The background absorbance was reduced by using freshly washed paper, by extracting chromatograms while they were still damp, by using a minimum volume of chloroform, and by evaporating the extracts immediately under nitrogen. Evaporation of washed commercial chloroform revealed a negligible residue. Where significant residues are obtained, the commercial chloroform should be redistilled or otherwise purified. The stopcocks were wetted with water only; other lubricants were avoided. All containers were rinsed with a suitable solvent before use. Background absorbance was greatly increased if ethyl alcohol was used as a rinse or as a stabilizer of the chloroform extracts. When the washed sheets were allowed to stand for days or weeks before use, the absorbance of paper blanks was increased.

Some estimations of the recoveries of the chromatographed cortisone and hydrocortisone were made by comparisons with the direct standards. The results of these analyses are given in Table II. Recoveries of 90 to 105% of the applied steroids were obtained. The lowest recoveries were obtained with amounts of 50 to 100 mcg. For best qualitative and quantitative results, it is recommended that amounts of 180 mcg. or greater of the steroids be used.

Paper chromatography of the hydrocortisone tablet extract revealed two ultraviolet absorbing zones. The more polar zone (2.0–4.4 cm.) corresponded to hydrocortisone in mobility. The less polar zone (9.3–11.7 cm.) had a mobility similar to 17-hydroxy-11-desoxycorticosterone. The infrared spectra of the extracted materials were identical to the standards. Quantitative measurements were made of the chromatographed steroids and the corresponding standards. Hydrocortisone and 17-hydroxy-11-desoxycorticosterone were present in the tablets in amounts of 46.1 and 53.9%, respectively. Previous analyses of this tablet by column chromatography and infrared spectrophotometry (9) revealed 47.5% hydrocortisone and 52.5% 17-hydroxy-11-desoxycorticosterone.

The paper chromatogram of the barbiturate fraction revealed three ultraviolet absorbing zones with mobilities similar to those of phenobarbital (2.0–3.8 cm.), butabarbital (4.1–6.1 cm.), and pentobarbital (6.4–8.8 cm.). The mobilities were dependent on the amount of immobile phase used to impregnate the paper. The infrared spectra of the paper chromatogram extracts were identical to those of the corresponding standards. The recoveries (Table III) agreed within 3.8% with the declared amounts.

The paper chromatogram of the sulfacetamide sample revealed two ultraviolet zones which corresponded in mobility to those of standard sulfacetamide (1.6–3.4 cm.) and sulfanilamide (0.0–1.6 cm.). The identities of these components were confirmed by comparing their infrared spectra with those of standard sulfacetamide and sulfanilamide. Sulfacetamide and sulfanilamide constituted 70.1 and 17.0%, respectively, of the total powder (Table III).

From these results, the general usefulness of the combined method is apparent. To the rapid and efficient paper chromatographic separation is added the specificity of the infrared spectra. The method should be adaptable to a variety of compounds. With modifications, such as the use of guide strips for color development, the study of ultraviolet transparent compounds could be made.

It is possible that improved qualitative and quan-

TABLE I.—MOBILITIES OF CORTISONE AND HYDROCORTISONE[a]

Sample No.	Hydrocortisone		Cortisone	
	Amount, mcg.	Mobility, cm.	Amount, mcg.	Mobility, cm.
1	50	1.8–4.4	50	5.6– 8.8
2	100	2.8–5.1	100	7.1–10.1
3	200	2.9–5.1	200	7.4–10.2
4	500	2.2–4.4	500	6.4– 9.3
5	1,460	2.1–5.1	186	7.7–10.3
6	92	2.6–5.0	1,000	7.0–11.4

[a] The mobility values give the distances of the leading and following edges of the zones from the starting line.

TABLE II.—RECOVERIES FROM CHROMATOGRAMS OF MIXTURES

Sample No.	Cortisone			Hydrocortisone		
	Applied, mcg.	Found, mcg.	Recovery, %	Applied, mcg.	Found, mcg.	Recovery, %
1	50	46	92.0	...	...	...
2	100	90	90.0	100	93.7	93.7
3	186	189	101.6	1,460	1,436.0	98.4
4	1,000	1,050	105.0	100	99.6	99.6

TABLE III.—RECOVERIES FROM COMMERCIAL PREPARATIONS

Sample	Active Ingredients	Declared, mg./Sample	Found	
			mg.	%
1	Hydrocortisone	20.00	9.22	46.1
	17-Hydroxy-11-desoxycorticosterone	0.00	10.78	53.9
2[a]	Phenobarbital sodium	2.67	2.77	103.7
	Butabarbital sodium	2.67	2.57	96.2
	Pentobarbital sodium	2.67	2.77	103.7
3	Sulfacetamide	100.00	70.10	70.1
	Sulfanilamide	0.00	17.00	17.0

[a] Pentaerythritol tetranitrate was present as 101.15% of declared amount.

titative results can be obtained on amounts less than 100 mcg. by using especially purified solvents and paper. Additional purification steps would be required in the analysis of compounds from biological media. It is anticipated that this method will be useful in the analyses of compounds from varied origins. Instruments equipped with beam condensers can be used if the aforementioned differences in beam sizes are considered.

SUMMARY

Cortisone and hydrocortisone were extracted from paper chromatograms and identified by their infrared spectra. The range of recoveries of amounts of 50 mcg. to 1.5 mg. was 90 to 105%. Analyses of commercial preparations, which contained corticosteroids, barbiturates, or sulfonamides, were achieved by paper chromatography and infrared spectrophotometry.

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Relationship Between *in Vitro* Disintegration Time and *in Vivo* Release of Vitamins from a Triple-Dose Spaced-Release Preparation

By A. B. MORRISON, C. B. PERUSSE, and J. A. CAMPBELL

Studies were conducted on the relationship between *in vitro* disintegration time and *in vivo* release of riboflavin, thiamine, and niacinamide in a multivitamin product consisting of three component spheres which disintegrated at different times. *In vitro* disintegration times were determined using apparatus and fluids described in U.S.P. XVI. None of the three vitamins was fully available in the intact product, as measured by the rates of urinary excretion of riboflavin, thiamine, and N′-methylnicotinamide after dosing human subjects. Spheres which showed incomplete availability as measured by urinary excretion of riboflavin were also relatively unavailable when excretion of thiamine or N′-methylnicotinamide was used as the criterion of adequacy. No evidence was obtained that the delayed disintegration of some of the spheres produced sustained release properties *in vivo*. One of the spheres was recovered essentially intact from the feces of two subjects. It was concluded that measurement of riboflavin excretion provides a valid indication of physiological availability of vitamin preparations and that limits for *in vitro* disintegration time based on riboflavin excretion are also applicable to other vitamins.

EARLIER work from this laboratory (1, 2) demonstrated that sugar coated tablets must disintegrate within 60 minutes (30 minutes in simulated gastric juice and 30 minutes in simulated intestinal juice) in order to show full availability of riboflavin. In additional studies (3), it was concluded that enteric coated riboflavin preparations should withstand the action of simulated gastric juice for 60 minutes and then disintegrate within 30 minutes in simulated intestinal juice, in order to show full availability of riboflavin. Little information is available on the relationship between *in vitro* disintegration time and physiological availability for other vitamins. The appearance on the market of a new "spaced-release" vitamin preparation offered an opportunity to examine the validity of proposed limits for disintegration time, using other vitamins in addition to riboflavin. The present report deals with the results of these studies.

METHODS

The product was purchased on the retail market in the United States and consisted of three "spaced-release" spheres contained within a gelatin capsule. Each sphere contained 3.33 mg. of thiamine hydrochloride, 1.67 mg. of riboflavin, and 16.7 mg. of niacinamide; one-third of the total dose of each vitamin in the product. One sphere was designed to release its ingredients immediately, whereas the second and third spheres were designed to release the vitamins contained therein approximately 3 and 6 hours later, respectively. The three spheres differed from each other, therefore, only *in vitro* disintegration time.

Physiological availability values for riboflavin, thiamine, and niacinamide were determined with the intact preparation and each of the three spheres. The procedure used to determine physiological availability was the same as that described previously (4, 5). Riboflavin, thiamine, and N′-methylnicotinamide levels were determined in the urine of 5 to 7 normal male subjects before dosing and after administration of standard or test preparations. The amounts obtained in the urine after dosing were corrected by subtracting the appropriate blank values determined on the urine of the same subjects. The blanks were determined over several days, to reduce the effect of daily variation. The subjects received doses of 5 mg. of riboflavin or 50 mg. of niacinamide in rapidly disintegrating standard tablets, as well as in the intact product and its component spheres. Because each sphere contained only one-third of the total dose in the product, it was necessary to give three spheres of each type in this portion of the studies. To provide a valid comparison with the rates of excretion of riboflavin and N′-methylnicotinamide after giving the intact preparation, the subjects also received the standard doses of riboflavin and niacinamide in divided dose form, as described previously (4, 6).

In the thiamine studies, the dose was only one of each sphere, and the resultant urinary excretion of thiamine was compared with that found after giving 3.33 mg. of the vitamin in solution. The dose was intentionally kept low because of the fact that large doses of thiamine are not absorbed efficiently (5, 7). The availability of thiamine in the intact product was determined by comparing urinary excretion of thiamine after giving 10 mg. of the vitamin in divided doses with that found after giving the product. It was necessary to use the divided dose standard for this purpose because of previous findings (5) that administration of 10 mg. thiamine in divided dose form markedly increased urinary excretion of the vitamin over that found when the same total dose was given at one time.

Received September 6, 1961, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.
Accepted for publication October 16, 1961.

Riboflavin and thiamine in the product and in urine were determined by the U.S.P. XVI fluorometric procedure (8) and by the thiochrome method of Mickelsen, *et al.* (9), respectively. Niacin in the product was determined by the U.S.P. XVI procedure (8), whereas urinary N′-methylnicotinamide was determined by the fluorometric procedure of Pelletier and Campbell (10). Curves of net rate of excretion were plotted on semilogarithmic paper, as suggested by Swintosky, *et al.* (11).

In vitro disintegration times for the three spheres were determined using the apparatus and fluids described in U.S.P. XVI (8). Disks were used in both the gastric and intestinal juice phases of the test. The spheres were immersed in simulated gastric fluid for 30 or 60 minutes and the remainder of the time in simulated intestinal fluid. Disintegration times reported were means of two separate tests on six spheres each.

Sphere R was given to two subjects and the feces were collected for a period of 48 hours after dosing. Undissolved spheres were recovered from the feces and analyzed for thiamine, riboflavin, niacinamide, folic acid, and ascorbic acid. With the exception of folic acid, which was analyzed by the procedure of Pelletier and Campbell (12), procedures given in U.S.P. XVI (8) were used for these analyses.

RESULTS

The *in vitro* disintegration times of the various spheres are summarized in Table I. Sphere O, designed to release immediately the vitamins contained therein, had the shortest disintegration time, whereas spheres Y and R, which were designed to release their vitamins approximately 3 and 6 hours after ingestion, had disintegration times which were much longer. The length of time in simulated gastric juice did not significantly affect the time required for disintegration in simulated intestinal juice.

Mean physiological availability values are shown in Table I and curves of excretion rate are given in Figs. 1-3. The percentage urinary excretion values for the standard single doses of riboflavin, thiamine, and niacinamide were 58, 14, and 19%, respectively. None of the vitamins was fully available in the intact product. Thiamine and niacinamide were fully available in sphere O, but riboflavin showed somewhat reduced availability in this component. Riboflavin and niacinamide showed similar low availability values in sphere Y, whereas thiamine, although not fully available, gave a higher value. All three vitamins showed low availability in sphere R.

Values found for thiamine with sphere Y showed considerable intersubject variation due, in part, to the fact that one subject was consistently unable to obtain any thiamine from this component. This subject was also unable to obtain any thiamine from sphere R.

The curves of excretion rates (Figs. 1–3) showed that sustained urinary excretion of riboflavin, thiamine, and N′-methylnicotinamide was obtained by giving the vitamins in divided doses. The intact product, however, showed no evidence of sustained release properties, as measured by urinary excretion of the vitamins tested. As shown in Fig. 1, the maximum rate of excretion of riboflavin after administration of the standard was higher than that found with the intact product or its component spheres. Spheres O and R showed maximum riboflavin excretion 2 hours after dosing, whereas the peak of excretion did not occur until 6 hours after ingestion of sphere Y.

The rate of excretion of N′-methylnicotinamide after ingestion of sphere O was very similar to that obtained with the standard (Fig. 2). Spheres Y and R, however, gave very low excretion curves. As with riboflavin, sphere Y gave peak excretion of N′-methylnicotinamide 2–4 hours later than did the other spheres.

As shown in Fig. 3, the rate of excretion of thiamine after dosing with sphere O was similar to that obtained with the standard. The curves for spheres Y and R were irregular and indicated low availability of the products.

The results of the fecal recovery studies are shown in Table II. Both subjects who consumed pellets of sphere R for this study recovered them from the feces. The amounts of niacinamide and folic acid obtained in spheres which passed through the gastrointestinal tract exceeded those claimed on the label. As well, 90% of the riboflavin, 84% of the thiamine, and 78% of the amount of ascorbic acid claimed on the label were found in spheres which were recovered from the feces.

DISCUSSION

The data again (13) emphasize the fact that processes which interfere with the disintegration or solution of a vitamin product in the gastrointestinal tract may have a marked effect on availability of vitamins. If the spheres are considered simply as plain coated tablets with varying disintegration times, they provide further evidence for the validity of a 1-hour limit for the *in vitro* disintegration time of vitamin preparations. Riboflavin tended to be somewhat less available in the various spheres than were the other two vitamins perhaps, in part, because of reduced solubility. Nevertheless, the results indi-

Table I.—Physiological Availability of Three Vitamins in a Spaced-Release Multivitamin Preparation and Its Component Spheres

Preparation	*In Vitro* Disintegration Time, min.			Availability, % ±S.E.		
	Gastric Juice	Intestinal Juice	Total	Riboflavin	Thiamine	Niacinamide
Intact product	..	...	...	38 ± 6.0	43 ± 5.9	64 ± 10.0
Sphere O	25	...	...	77 ± 5.1	98 ± 13.7	122 ± 14.7
Sphere Y	30	57	87	20 ± 1.9	73 ± 21.6	27 ± 6.4
	60	54	114	...	...	...
Sphere R	30	138	168	13 ± 4.5	28 ± 5.0	29 ± 7.8
	60	134	194	...	...	...

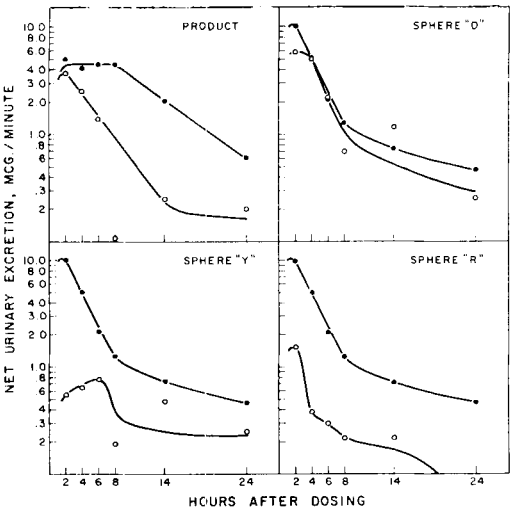


Fig. 1.—Urinary excretion curves for riboflavin in product or its component spheres O—O—O compared with curves for riboflavin standard ●—●—●.

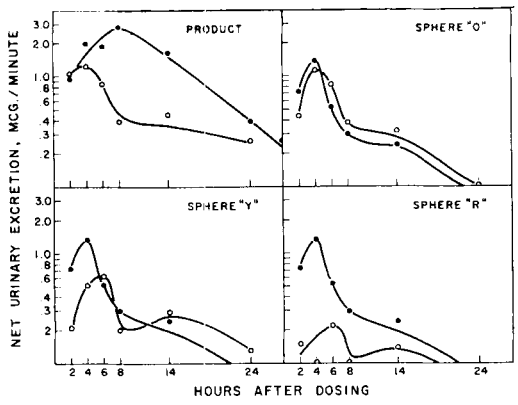


Fig. 2.—Urinary excretion curves for thiamine in product or its component spheres O—O—O compared with curves for thiamine standard ●—●—●.

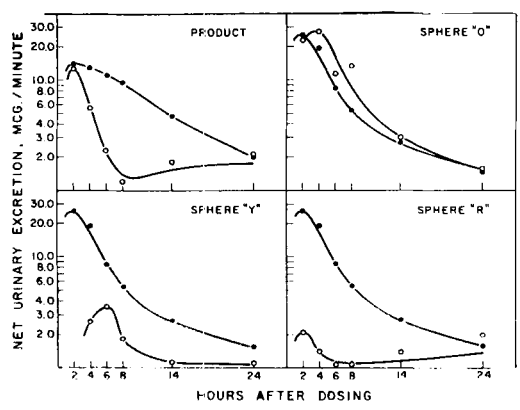


Fig. 3.—Urinary excretion curves for N′-methylnicotinamide for product or its component spheres O—O—O compared with those for niacinamide standard ●—●—●.

Table II—Vitamin Content of Sphere R Recovered from Feces

Vitamin	Amount Recovered, % of Label Claim
Riboflavin	90
Thiamine	84
Niacinamide	140
Folic acid	118
Ascorbic acid	78

cated that spheres which showed incomplete availability as measured by urinary excretion of riboflavin were also unavailable when excretion of thiamine or N′-methylnicotinamide was used as the criterion of adequacy. This finding would appear to allay the fears of Endicott and Kirchmeyer (14) that vitamins more soluble than riboflavin might yield an entirely different relationship between *in vitro* disintegration time and physiological availability.

The time limit given in U.S.P. XVI (8) for plain coated decavitamin tablets is 30 minutes in simulated gastric juice plus 2 hours in simulated intestinal juice. Sphere R would not pass such a test, but sphere Y would. The latter sphere, however, showed inadequate availability of all three vitamins tested. It is obvious that riboflavin, thiamine, and niacinamide in coated tablets which pass the U.S.P. test may not be fully available to the body.

Because of the resistant coatings used, it might appear more appropriate to consider spheres Y and R as enteric coated preparations. In a previous communication (3) it was suggested that enteric coated products containing riboflavin should withstand simulated gastric juice for at least 60 minutes and should then disintegrate within 30 minutes in simulated intestinal juice, to ensure full availability *in vivo*. The *in vitro* disintegration times for spheres Y and R exceeded this time limit, and both spheres showed incomplete availability of all three vitamins tested. These results suggest that the proposed time limit is also applicable to enteric coated preparations containing other vitamins.

If decavitamin preparations are enteric coated, the time limit given in U.S.P. XVI (8) is 5 hours (1 hour in simulated gastric juice plus 4 hours in simulated intestinal juice). Sphere R, which would have passed the U.S.P. XVI test, was recovered essentially intact from the feces of human subjects. These results indicate that the present U.S.P. time limit for enteric coated decavitamin preparations is too long, and that products which pass the test may not be available to the body.

The results of the fecal recovery studies indicated that tablets with an *in vitro* disintegration time similar to that of sphere R would exhibit very low availability not only of riboflavin, thiamine, and niacinamide, but also of folic acid and ascorbic acid. These findings suggest that the relationship between *in vitro* disintegration time and physiological availability for folic acid and ascorbic acid is similar to that obtained with riboflavin, thiamine, and niacinamide.

No evidence was obtained that the intact preparation possessed sustained-release properties, nor that the total dose was released at three separate times. Sphere R, which had the longest *in vitro* disintegration time, was recovered almost intact from the

feces of two subjects. It is obvious that the vitamins in this component of the product were largely wasted and that the sphere could not exert desired effects *in vivo*. The results obtained do not support claims that the product provides improved nutritional benefits with less loss of vitamins, and are in agreement with similar findings obtained previously with other sustained release vitamin preparations (4, 5). Furthermore, the data again (4) emphasize that it is most difficult to formulate vitamin preparations in true sustained-release form.

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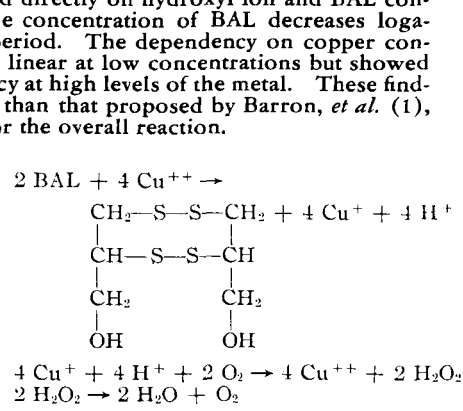
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Kinetics of Copper Catalyzed Oxidation of 2,3-Dimercapto-1-propanol by Molecular Oxygen

By EDWARD G. RIPPIE† and TAKERU HIGUCHI‡

The rate of disappearance of the sulfhydryl groups of 2,3-dimercapto-1-propanol (BAL) in aqueous solutions in the presence of molecular oxygen and catalytic quantities of copper has been shown to depend directly on hydroxyl ion and BAL concentrations and on oxygen pressure. The concentration of BAL decreases logarithmically following a short induction period. The dependency on copper concentration was found to be approximately linear at low concentrations but showed an apparent limiting zero-order dependency at high levels of the metal. These findings suggest that some mechanism other than that proposed by Barron, *et al.* (1), must be responsible for the overall reaction.

BARRON, *et al.* (1), studying the oxidation of (BAL) by dissolved molecular oxygen in aqueous solutions in the presence of copper, manometrically, have reported that BAL and and similar dithiols apparently do not undergo oxidation by free oxygen in the absence of certain metal ions and that any observed oxidation in the apparent absence of these catalysts is due to residual trace quantities of metals (probably copper). They suggest a mechanism involving three stoichiometric reactions



Every dithol molecule oxidized, according to this mechanism, involves reaction with copper. Present findings based on iodometric determination of residual sulfhydryl groups appear to be in partial conflict with these suggestions. A possible alternate route is presented.

EXPERIMENTAL

Reagents.—Reagent grade mono- and dipotassium phosphate; 0.1 *N* iodine solution; 0.1 *N* thiosulfate solution; oxygen U.S.P.; catalase (Worthington); water redistilled from all glass apparatus; 2,3-dimercapto-1-propanol containing not more than 0.93% 1,2,3-trimercaptopropane.

Received September 11, 1961, from the School of Pharmacy, University of Minnesota, Minneapolis 14.
Accepted for publication September 28, 1961.
Based in part on a thesis submitted by E. G. Rippie to the Graduate School, University of Wisconsin, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
This investigation was supported in part by a grant from the Research Committe of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
The authors wish to acknowledge the help given to us by Dr Albert A. Kondritzer, Chief of the Physiological Chemistry Branch, Army Chemical Center, Md., in carrying out these studies.
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feces of two subjects. It is obvious that the vitamins in this component of the product were largely wasted and that the sphere could not exert desired effects *in vivo*. The results obtained do not support claims that the product provides improved nutritional benefits with less loss of vitamins, and are in agreement with similar findings obtained previously with other sustained release vitamin preparations (4, 5). Furthermore, the data again (4) emphasize that it is most difficult to formulate vitamin preparations in true sustained-release form.

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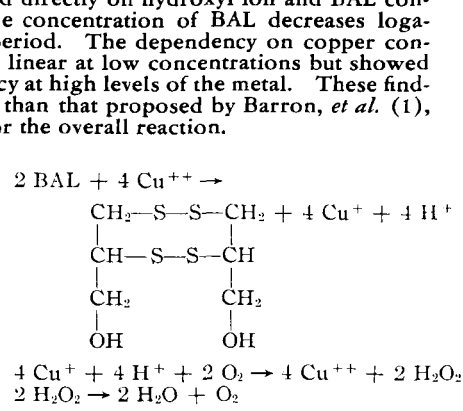
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Kinetics of Copper Catalyzed Oxidation of 2,3-Dimercapto-1-propanol by Molecular Oxygen

By EDWARD G. RIPPIE† and TAKERU HIGUCHI‡

The rate of disappearance of the sulfhydryl groups of 2,3-dimercapto-1-propanol (BAL) in aqueous solutions in the presence of molecular oxygen and catalytic quantities of copper has been shown to depend directly on hydroxyl ion and BAL concentrations and on oxygen pressure. The concentration of BAL decreases logarithmically following a short induction period. The dependency on copper concentration was found to be approximately linear at low concentrations but showed an apparent limiting zero-order dependency at high levels of the metal. These findings suggest that some mechanism other than that proposed by Barron, *et al.* (1), must be responsible for the overall reaction.

BARRON, *et al.* (1), studying the oxidation of (BAL) by dissolved molecular oxygen in aqueous solutions in the presence of copper, manometrically, have reported that BAL and and similar dithiols apparently do not undergo oxidation by free oxygen in the absence of certain metal ions and that any observed oxidation in the apparent absence of these catalysts is due to residual trace quantities of metals (probably copper). They suggest a mechanism involving three stoichiometric reactions



Every dithol molecule oxidized, according to this mechanism, involves reaction with copper. Present findings based on iodometric determination of residual sulfhydryl groups appear to be in partial conflict with these suggestions. A possible alternate route is presented.

EXPERIMENTAL

Reagents.—Reagent grade mono- and dipotassium phosphate; 0.1 *N* iodine solution; 0.1 *N* thiosulfate solution; oxygen U.S.P.; catalase (Worthington); water redistilled from all glass apparatus; 2,3-dimercapto-1-propanol containing not more than 0.93% 1,2,3-trimercaptopropane.

Received September 11, 1961, from the School of Pharmacy, University of Minnesota, Minneapolis 14.
Accepted for publication September 28, 1961.
Based in part on a thesis submitted by E. G. Rippie to the Graduate School, University of Wisconsin, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
This investigation was supported in part by a grant from the Research Committe of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
The authors wish to acknowledge the help given to us by Dr Albert A. Kondritzer, Chief of the Physiological Chemistry Branch, Army Chemical Center, Md., in carrying out these studies.
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Apparatus.—A water-jacketed, glass reaction vessel was fitted with a water-sealed stirrer, manometer, and leveling bulb for maintenance of oxygen pressure, and a tube dipping below the level of the thiol solution for removal of samples at various time intervals. This apparatus was used in the experiments which were run with the oxygen pressure equal to atmospheric pressure.

A glass pressure vessel was used at elevated oxygen pressures. The bomb was fitted with a heavy rubber diaphragm through which samples could be withdrawn periodically by means of a hypodermic syringe. A Beckman model G pH meter equipped with a thermostated cell was used to determine the pH of reaction mixtures at the various temperatures. Titrations were carried out with a 2-ml. microburet with an immersion tip.

Procedure.—All studies were carried out in solutions thermostated to ±0.1°. The ionic strength was maintained constant at 0.5 by phosphate buffer to eliminate salt effects. Measured quantities of BAL were dissolved in the buffer solution containing the appropriate quantity of copper sulfate. The system was then flushed with oxygen, sealed, and stirred with the stirrer, or shaker in the case of the pressure bomb, with sufficient violence to assure saturation with respect to oxygen. Samples were withdrawn periodically for analysis.

Residual sulfhydryl content was determined iodometrically. An aliquot of the mixture was quenched by pipetting a sample of the reaction mixture into 0.1 *N* iodine solution, the excess iodine being back-titrated with 0.1 *N* thiosulfate. The insoluble disulfide present during the titration did not interfere with the end point, which was taken as the disappearance of the iodine color since starch indicator did not work well in the presence of oxidized BAL. A precision of ±1 μl. was obtained with a microburet fitted with an immersion tip.

Since in preliminary experiments it was found that hydrogen peroxide formed during the reaction interfered with the iodine titration, catalase was added (200 units per ml.) to all subsequent reaction solutions. Keilin (2) has shown that although both blood and liver catalase preparations increase the rate of thiol oxidation, the concentration necessary is 10^5 times higher than that required for the catalytic decomposition of hydrogen peroxide at an approximately similar rate. While Webb (3) found up to 90% inhibition of catalase by BAL, the amount remaining was presumed to be sufficient to decompose any peroxide formed. The addition of catalase did not appreciably alter the chemical kinetics of BAL oxidation but permitted us to follow the reaction closer to completion without interference by peroxide.

RESULTS AND DISCUSSION

Order of the Reaction with Respect to BAL.—The rate of disappearance of sulfhydryl groups in the presence of copper from stirred agueous solutions of BAL under constant oxygen tension appeared to be complex functions of time as is evident from data presented in Fig. 1. There seemed to be an initial autocatalytic lag phase followed by an apparently first-order disappearance of SH groups from the solution. The exact form and extent of the induction phase could not be established easily since the

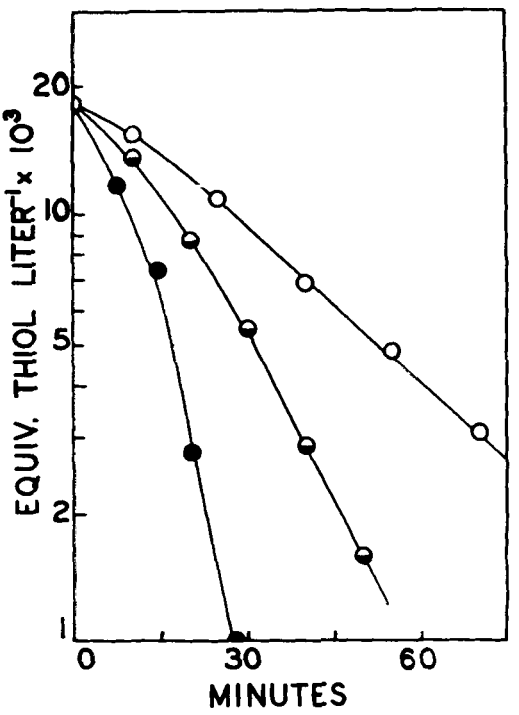


Fig. 1.—Semilog plots of thiol titer *vs.* time showing lag time. ○, 15°; ◒, 25°; ●, 35°; 2×10^{-5} *M* Cu(II); 760 mm. O_2 pressure; pH = 6.66.

experimental procedure employed did not permit determinations of an unambiguous zero time for the reaction. The induction period was also noted for systems followed manometrically, although Barron, *et al.* (1), do not report this.

The first-order phase of the observed reaction rate seemed to be truly first order. The slopes of the straight line portions such as are shown in Fig. 1 were found to be independent of initial BAL concentration within experimental error over a wide range of concentrations. This relationship was also found to be valid at different oxygen tensions as is evident from Fig. 2. For these runs, readings were taken after the initial phase so that only that part of the reaction which follows the first-order dependency is shown.

Effect of Oxygen Pressure.—The rate of the copper catalyzed oxidation of BAL appeared to vary directly with oxygen tension. This is evident in Fig. 3 where the first-order reaction rate constants calculated from the straight line portion from plots such as shown in Fig. 2 were plotted against pressure to yield a straight line passing through the origin. Numerical values of the rate constants and some of the experimental conditions are given in Table I.

For these runs, solutions of BAL were shaken violently in a glass reaction bomb under various oxygen pressures; aliquots were withdrawn and titrated periodically. Drop in oxygen pressure, due to consumption by the reaction and withdrawal of aliquots, introduced an estimated error of 2%. Pressures of oxygen were assumed to be partial pressures since the vapor pressure of water was negligible in this case.

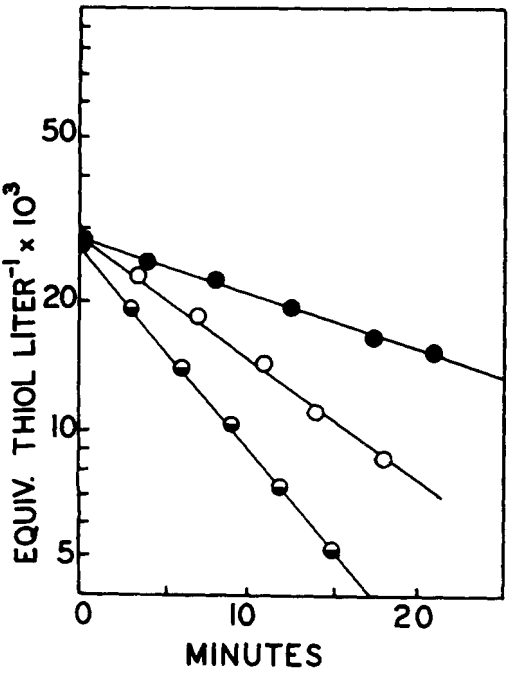


Fig. 2.—Plot showing first-order dependence on BAL after lag phase at several oxygen pressures; time "0" was taken arbitrarily at approximately 5 minutes following reaction initiation. ●, 760; ○, 1580; ◒, 2400 mm. O_2 pressure; 24.5°; pH = 6.66; 1×10^{-5} *M* Cu(II).

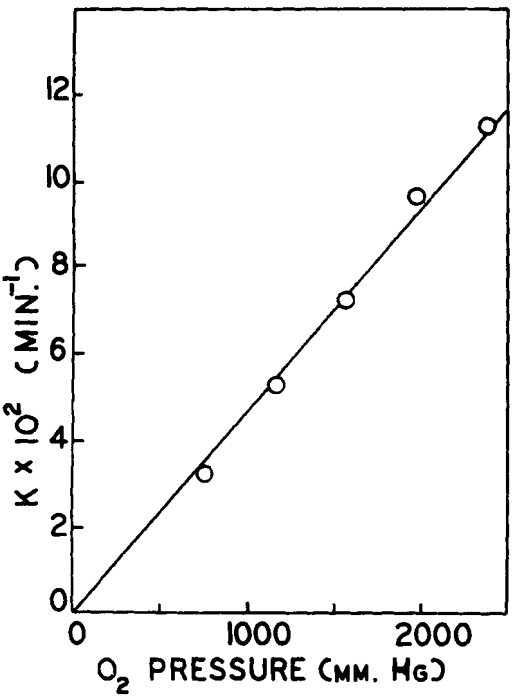


Fig. 3.—Plot showing first-order nature of BAL oxidation with respect to oxygen pressure in the presence of copper. Readings were taken at 24.5°; pH = 6.66; 1×10^{-5} *M* Cu(II).

TABLE I.—EXPERIMENTAL RATES AT VARIOUS COPPER CONCENTRATIONS AND OXYGEN PRESSURES AT pH 6.66

Temperature, °C.	Cu(II) Concn., *M*	O_2 Pressure, mm. Hg	$k \times 10^{-2}$ min.$^{-1}$
20.0	2×10^{-6}	760	1.07
20.0	5×10^{-6}	760	1.61
20.0	1×10^{-5}	760	2.65
20.0	2×10^{-5}	760	3.96
20.0	4×10^{-5}	760	5.21
24.5	1×10^{-5}	760	3.25
24.5	1×10^{-5}	1170	5.21
24.5	1×10^{-5}	1580	7.26
24.5	1×10^{-5}	1990	9.63
24.5	1×10^{-5}	2400	11.27

Effect of Hydrogen Ion Concentration and Temperature.—The effect of pH on the rate of BAL oxidation was determined at 15, 20, and 25°. Figure 4 shows the collected results of these determinations in which the logarithm of the rate in reciprocal minutes has been plotted against the pH determined at the individual temperatures. The lines, which have an average positive slope of 0.73, are essentially parallel.

Figure 5 shows the results of heat of activation determinations at three pH's. The average value of the heat of activation, ΔHa, as determined from the slopes, was 12.6 Kcal. per mole. No account was taken of the effect of temperature on oxygen solubility or on the degree of ionization of BAL

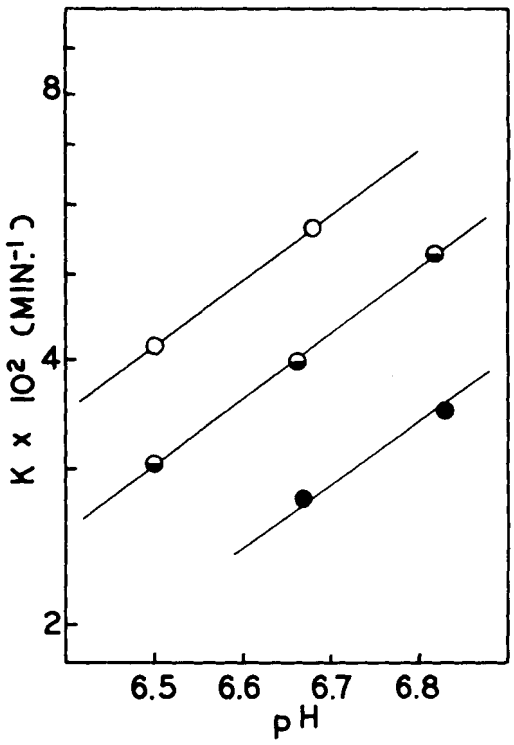


Fig. 4.—Semilog plots of first-order constants of BAL oxidation in reciprocal minutes against pH. The average slope of the lines is 0.73. Readings were taken at ○, 25°; ◒, 20°; ●, 15°; 1 atm. O_2 pressure; 2×10^{-4} *M* Cu(II).

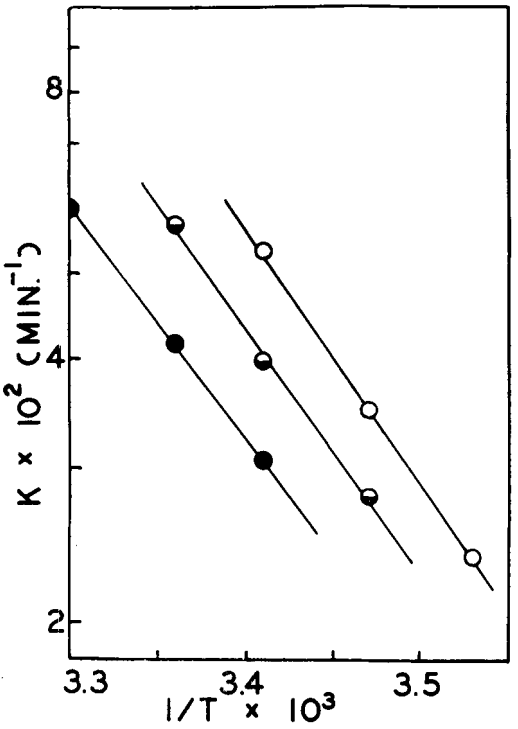


Fig. 5.—Typical Arrhenius plots obtained at different pH values. Solutions contained 2×10^{-5} *M* Cu(II); runs were made at ○, pH = 6.83, ◒, pH = 6.66, ●, pH = 6.50, under 1 atm. O_2.

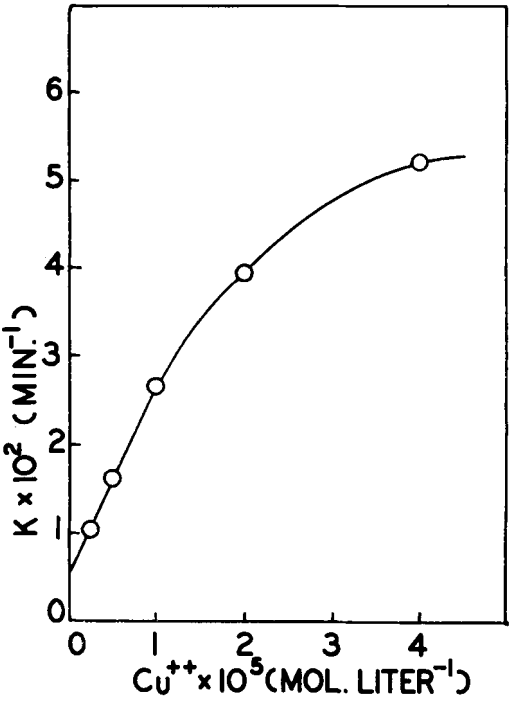


Fig. 6.—Plot showing linear copper dependency of first-order reaction rate constants at low copper concentration. Runs were made at 20°, pH = 6.66, under 1 atm. O_2.

in this calculation; therefore, the ΔHa given is the apparent heat of activation. The subtraction of the heat of solution of oxygen in water, −3.6 Kcal. per mole, from the apparent ΔHa yields a corrected value of 16.2 Kcal. per mole for the process. The heat of ionization of BAL, however, was not known and no correction has been made for its presumably small effect. Results of these experiments are given in Table II.

TABLE II.—EXPERIMENTAL RATES AT VARIOUS TEMPERATURES AND pH VALUES[a]

Temperature, °C.	pH	Rate constant × 10^2, min.$^{-1}$
10	6.83	2.36
15	6.83	3.49
15	6.67	2.77
20	6.82	5.29
20	6.66	3.96
20	6.50	3.05
25	6.68	5.63
25	6.50	4.14
30	6.48	5.88

[a] Cu(II) = 2×10^{-5} *M*; O_2 pressure = 760 mm. Hg.

Effect of Copper Concentration on Reaction Rate.—Rate determinations made in the presence of varying copper concentrations yielded data shown plotted in Fig. 6, which indicate a nonlinear relationship between the two. The extrapolated value of the rate constant at zero copper concentration agreed with the experimentally determined rate of oxidation when a copper-free solution of BAL was oxidized in the presence of 1×10^{-3} molar potassium cyanide. In Fig. 7, the reciprocal of the observed rate constant has been plotted against the reciprocal of the copper concentration. Extrapolation of the resulting straight line plot to infinite copper concentration indicates a limiting rate constant equal to 7.81×10^{-2} min.$^{-1}$.

The saturation effect has been previously noted in studies on the cysteine-cystine system by Elliott (4) who found that the rate of oxidation by molecular oxygen was proportional to cupric ion at low concentrations, after which the rate was found to approach a limiting value. No mention was made of a possible cause of this phenomenon.

Consideration of the Overall Copper Catalyzed Reaction.—No limiting oxidation rate at high copper concentration, as is actually observed, would be expected from a totally copper-mediated mechanism such as has been proposed (1), as any increase in copper concentration would accelerate the oxidation further. One might expect, moreover, a limiting rate with respect to BAL, especially since the overall rate is directly dependent on oxygen. At BAL concentrations substantially in excess of the copper concentration, a BAL-copper complex could be expected to limit the breakdown rate because its concentration would not change appreciably with a change in BAL concentration. It is also apparent that if oxidation of the dithiol was totally mediated by copper, the overall rate would be rate-limited by either the rate of reduction of Cu(II) or its regeneration. In the former case, the observed rate would be substantially independent of oxygen tension;

in the latter, the rate should be largely zero order with respect to BAL.

As a result of the present studies, the following alternate reactions are proposed as possible components of the overall reactions. We are at present unable to write the total mechanism because of the apparent great complexity of the system. These reactions are, however, suggested on the basis of the present studies. Since the exact forms of cupric and cuprous copper have not been established, copper is shown only as to its oxidation state in the following reactions

$$Cu(II) + B(SH)S^- \xrightarrow{k_1} Cu(I) + B(SH)S\cdot \quad \text{(Step 1)}$$

$$B(SH)S\cdot + O_2 \xrightarrow{k_2} BS_2 + HO_2\cdot \quad \text{(Step 2)}$$

$$HO_2\cdot + B(SH)S^- \xrightarrow{k_3} B(SH)S\cdot + HO_2^- \quad \text{(Step 3)}$$

$$3\,Cu(I) + 3\,H^+ + HO_2\cdot \xrightarrow{k_4} 3\,Cu(II) + 2\,H_2O \quad \text{(Step 4)}$$

$$Cu(II) + B(SH)S\cdot \xrightarrow{k_5} Cu(I) + H^+ + BS_2 \quad \text{(Step 5)}$$

If these reactions are considered to be representative of the principal reactions occurring after lapse of the lag time, several observations can be rationalized.

It can be seen that, when the copper concentration is such that step 5 occurs at a rate still insignificant compared with step 2, the rate is apparently independent of copper concentration. This was the case experimentally. Nord (5) in his studies on the autoxidation of cuprous chloride, reported rapid air oxidation of the cuprous ion at hydrogen ion concentrations of 0.1 *M* and above which, if extrapolated to neutrality, would predict extremely slow reaction rates. He also reported a pH-independent oxidation of cuprous ion at hydrogen ion concentrations below 0.1 *M*. If this rather slow reaction were assumed to occur at neutrality and if cupric ion were the only oxidant of BAL, it would also fail to account for the rapid oxidation of the sulfhydryl compound noted in the present work.

The illustrated partial mechanism agrees with the experimentally observed first-order dependency on BAL. However, a discrepancy may be noted between the expected and observed slopes when the overall rate constants are plotted *vs.* pH. If the dissociation of the B(SH)S· species to $BS_2^{\cdot-}$ is necessary before the reaction with cupric ion, the reaction 5 will be a function of the pH and result in a negative deviation from slope 1.0, as observed. Hydrogen peroxide as such was not thought to contribute significantly to the reaction rate since no detectable

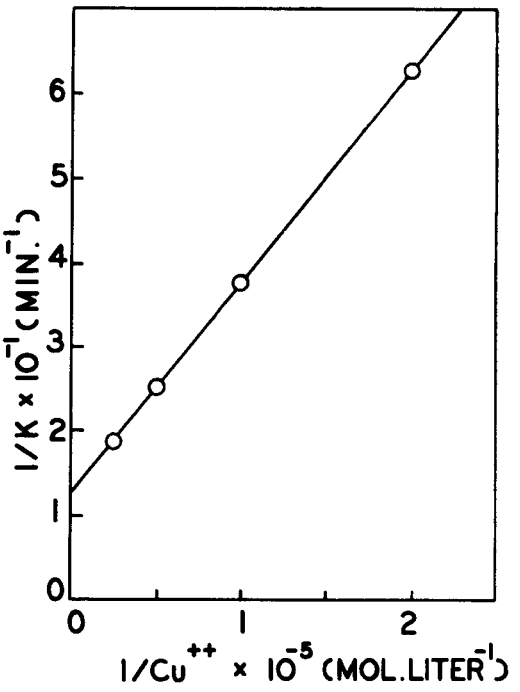


Fig. 7.—Plot of reciprocal first-order rate constant against reciprocal copper concentration, indicating limiting rate constant of 7.81×10^{-2} (min.$^{-1}$). Runs were made at 20°, pH = 6.66, under 1 atm. O_2.

change in the overall rate constant was found upon addition of catalase to the system.

Although this mechanism appears to be consistent with the experimental evidence, it is not necessarily unique since alternate mechanisms may be equally successful in explaining the experimental findings Moreover, the chemical equations suggested here should not be considered as the only ones occurring. There is a possibility that reactions, kinetically indistinguishable from those suggested, may take part in the oxidative breakdown.

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Effects of Centrally-Acting Drugs on Human Motor and Psychomotor Performance

By DUANE G. WENZEL and CHARLES O. RUTLEDGE

The dose-effect and duration of action of several stimulants and tranquilizers were determined using motor and psychomotor performance tests with humans. Three doses of each of the following drugs were employed: placebo, caffeine, *d*-amphetamine, phenmetrazine, methyl phenidyl acetate, phenobarbital, chlorpromazine, meprobamate, and oxanamide. Phenmetrazine and methyl phenidyl acetate were also studied with additional tests designed to measure fatigue. Deanol and placebo groups were tested weekly for 8 weeks of daily administration crossing treatments at 4 weeks. The tranquilizers did not affect performance as measured by the tests employed, while all stimulants produced some improvements in all tests. Phenmetrazine and methyl phenidyl acetate were the most promising central stimulants tested. These were found to mitigate fatigue by elevation of the initial performance level and by decreasing the time-related decrement. The action of deanol was equivocal with some indication of an interaction between the drug and subject interest.

PROBLEMS inherent in the guidance of aircraft or anticipated spacecraft have increased to the point where, despite refinements in instrumentation, they have begun to exceed man's physiologic and psychologic limits. The obvious solution to the problem is to alter man himself. This might be accomplished through education and training and (or) through the mediation of centrally acting drugs. While it is perhaps self-evident that the improved control of rapidly moving objects is a worthwhile objective, an even more important objective could be the production of periods of enhanced efficiency under conditions of stress or fatigue. Although many studies have been conducted to determine the effects of centrally acting drugs upon human performance, most of these have been clinical in nature, lack proper controls, or they have been oriented toward the treatment of mental illness. It is therefore difficult to compare the effects of such drugs upon normal performance.

The minimum requirements for the proper evaluation of a drug affecting performance would seem to be that: the temporal and dose effects of the drug must be measured with several types of performance; the subjects employed must be adequate in number and be reasonably uniform, physiologically and psychologically; and the experimental design must provide adequate controls and employ suitable techniques to avoid bias.

In the studies which follow every effort was made to meet these requirements, although, as noted in the discussion, it became necessary to reach certain compromises dictated by the limitations of time.

EXPERIMENTAL

Central Stimulant Study.—Subjects consisted of 15 volunteer, male, college students between the ages of 22 and 27, and weighing between 145 and 185 pounds. All were experimentally naive prior to testing and were of approximately the same intellectual capacity as indicated by the rank in their class. Subjects were instructed in the test procedure by means of detailed printed instructions and an oral orientation period. They were then repeatedly tested in practice sessions until a stable level of performance was achieved for each test. Treatments consisted of the following drug doses: placebo; caffeine, 100, 200, and 300 mg.; *d*-amphetamine, 2.5, 5, and 10 mg.; phenmetrazine, 12.5, 25, and 50 mg.; and methyl phenidyl acetate, 5, 10, and 15 mg. Doses were selected by bracketing what was thought to be the amount of the drug which would induce definite physiological and psychological effects.

All testing was performed between 1:00 and 5:00 p.m. At approximately 12:00 p.m., 1 hour prior to the administration of the drug, subjects ate a light lunch consisting usually of a sandwich, a glass of milk, and some fruit. They were instructed to refrain from drinking caffeinated beverages and from smoking following breakfast. Five subjects were each tested four times in one afternoon at hourly intervals. The first test for all subjects, or control, was conducted one hour after lunch. Immediately

Received April 28, 1961, from the University of Kansas, School of Pharmacy, Lawrence.

Accepted for publication August 7, 1961.

Abstracted from a portion of the data contained in a thesis submitted to the Graduate School of The University of Kansas by Charles O. Rutledge for the degree of Master of Science in Pharmacology.

This work was supported by a contract between the Office of Naval Research and The University of Kansas.

The authors gratefully acknowledge the medical supervision of the drug administration by Dr. R. L. Hermes. Dr. Edward L. Wike of the Department of Psychology is thanked for his assistance with the statistical analysis. Paul W. Davis served as a technical assistant. Thanks are extended to the following individuals and firms for the drugs used in this study: Edward E. Frost, Jr., Smith Kline and French Laboratories for the *d*-amphetamine (Dexedrine); Joseph Marrus of Geigy Pharmaceuticals for the phenmetrazine (Preludin); Dr. Frank A. Travers of Ciba Pharmaceutical Products for the methyl phenidyl acetate (Ritalin); Dr. R. C. Pogge of the Wm. S. Merrell Co. for the oxanamide (Quiactin); and Dr. Adolph Surtshin of Riker Laboratories for the deanol (Deaner).

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

following this test a No. 1 pink capsule containing the drug was administered with a glass of water. The selection of the drug-dose was randomized and double-blind conditions were maintained throughout the study. Testing was repeated three times at hourly intervals following the first test and the administration of the drug.

As each subject was to be tested with each of 13 drug or placebo doses, it was anticipated that boredom or disinterest might influence the results after the novelty of early testing wore off. In an attempt to minimize this possibility and yet not introduce new factors of variability, two techniques were employed in order to maintain positive motivation. Competitive motivation was used to the extent that the three highest individual scores for each test were posted weekly with the previous record score. No further efforts were made to induce individual or group competition because such methods have been found to create variable secondary effects (1). Self-competition related to knowledge of performance (2) was difficult to induce because of the changing drug factor. Subjects were not allowed to observe another's performance nor did they receive information concerning their own scores except as indicated. The second measure designed to stimulate motivation was the encouragement of subjects to maintain a record of their own subjective responses to each treatment. They were informed that at the conclusion of the study they would be provided with their own test results and the drugs employed at each period. This procedure provoked considerable speculation and maintained interest in the effects of each treatment. Subjects were not informed of the drugs that were to be employed other than that moderate doses of nontoxic agents were to be used.

All experiments were conducted in an isolated laboratory equipped specifically for this purpose. The room was well lighted with overhead fluorescent lamps as the sole source of light. Room temperature was held between 70 and 72°F. Ambient noise consisted of the sound of the fan motor in the counter and the motors of the tracking test. These were kept running continuously throughout the entire afternoon of testing and provided a low steady noise level sufficient to mask external sounds. Conversation between subject and experimenter was not permitted except for necessary signals and instructions.

Tests

Tapping Rate.—A microswitch with a roller contact was used as a key in the tapping tests. The switch had a lever arm of 1 in., a pretravel distance of $^1/_4$ in., and required an actuating force of 140 Gm. The roller materially reduced finger friction and in the preliminary testing gave more consistent results than similar keys without the roller. It was mounted at table height so that the subject sat facing the switch with his arm comfortably bent at the elbow and his forefinger on the key. The thumb and remaining fingers were folded beneath the hand and kept in constant contact with the table. This precaution was necessary in order to avoid actuation of the switch with the muscles of the hand rather than of the finger. Use of the hand enabled subjects to obtain higher scores, especially in the 60-second test in which finger fatigue became marked. Conformity with this provision was assured by careful preinstruction and continual observation by the experimenter.

The key was wired into an electric timing circuit in such a manner that the first tap activated an adjustable timer previously set for the desired period over which the tapping rate was to be measured. Starting the timer simultaneously closed a relay connecting the key to an electric four-digit counter. The stopping of the timer at the end of the preset interval opened the relay and discontinued the counting. In this manner subjects could begin tapping whenever they felt that they were ready and the total count for the specified period was recorded automatically. The circuit for this counter system is shown in Fig. 1.

One objective of the tapping test was to determine the maximum speed at which a voluntary movement could be repeated. For this purpose the test must be conducted over an accurately measurable time interval yet one that is short enough to prevent fatigue. A 5-second period was employed. It was repeated four times at each of 4 hourly test periods.

The rate of tapping may also be used to observe the effect of fatigue on the motor activity of the discrete group of muscles controlling finger movement. For this purpose the test was conducted over a 60-second interval. Because of the problem of cumulative fatigue, it was possible to perform this test only twice in any one period, before and after an 8-minute interval during which the other tests were being performed.

Simple Visual Reaction Time.—The visual stimulus for this test consisted of the light from a 120-v., 6-watt, Sylvania S6 lamp mounted in a holder behind a 1-in., clear, jeweled lens. The lamp was placed in a cabinet about 3 ft. from the subject and was 6 in. above the desk top. The subject was seated before the light with his index finger on a snap-action switch having a pretravel distance of $^1/_{16}$ in. With the subject in position, the experimenter, seated behind the cabinet, gave a verbal signal of "ready," at which time he simultaneously closed a switch in a time-delay circuit. This circuit was previously and at random varied by the experimeter between time intervals of from 2 to 5 seconds. At the completion of the time-delay, the circuit to the lamp and a Beckman time interval meter model 7250C were activated, turning on the light and starting the counter. When the snap switch was then closed by the subject, the timer was stopped and the time was measured to 0.1 msec. The circuit diagram of this apparatus is presented in Fig. 1. Four replicates of this test were conducted at each test period.

Complex Reaction Time.—This test was modified from one described by Borkenstein (3) which was termed "choice reaction." The apparatus for this test consisted of a 9 × 9 inch panel of opalescent plexiglas mounted on the sloping front of the cabinet containing the simple reaction time light. The panel was divided into sixteen 1.5 in. square spaces in a 4 by 4 arrangement. Each space had a black 1-in. number arranged in the order from top row to bottom row: 9-6-3-5, 6-2-4-7, 7-8-9-8, and 5-2-3-4. These numbers were on the reverse side of the plexiglas panel and were not visible until individual lights, mounted separately behind each number, were lit. The lights could be lit in either of two manners. First, they could be lit two lights at a time in a con-

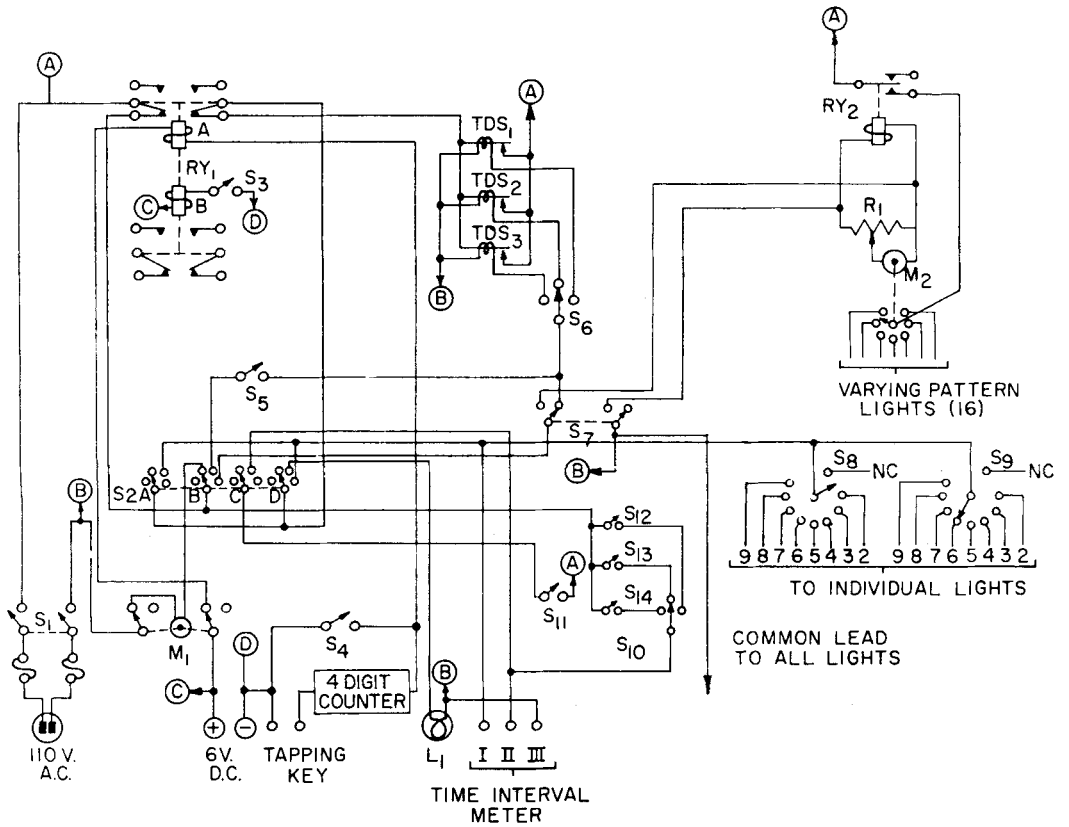


Fig. 1.—Circuit diagram of the unit controlling tapping tests and simple and complex reaction times. All switches except $S_{11, 12, 13, \text{and } 14}$ are mounted on the rear of the cabinet. S_1, SPDT switch controlling 110 v. a.c. power. S_2, 4P 4-position switch controlling test function; A, B, C, and D represent switch gangs; positions are 1, 2, 3, and 4 beginning to the left of the pole and proceeding clockwise; all gangs are shown in position 2; position 1, off; position 2, tapping rate; position 3, visual reaction time; and position 4, complex reaction time. S_3 and S_4, momentary SPDT, NO switches controlling RY_1 relay position. S_5, SPDT off-on time delay switch. S_6, SP 3-position switch for selection of time-delay circuit. S_7, DP 2-position switch to turn on motor switching varying pattern lights (M_2) and lights through relay RY_2 or to activate one of three time-delay switches ($TDS_{1, 2, 3}$). S_8 and S_9, SP 10-position switches controlling lighted number pair of complex reaction time test. S_{10}, SP 3-position switch for selection of odd, even, or odd-even pairs, as indicated by positions of S_8 and S_9. S_{11}, SPST momentary switch thrown by subject to complete visual reaction time. S_{12}, S_{13}, and S_{14}, SPST momentary switches thrown by subject to complete odd, even, or odd-even pairs for complex reaction time. RY_1, SP, 2-position, 2-section latching relay controlling a.c. power. RY_2, SP, 2-position relay to supply power to motor-driven switch prior to activating time delay switches by S_7. $TDS_{1, 2, 3}$, Amperite 2, 3, and 5-second delay relays. M_1, Industrial Timer Corp., TDAF-60-second series timer. M_2, varying pattern lights switch motor. I, Start position on Beckman time interval meter. II, Stop position. III, Common. Circled letters represent common connections.

tinuously varying pattern by means of a motor-driven rotating multicontact switch. Secondly, any two selected numbers could be lit by the experimenter following a time delay of from 2 to 5 seconds. The test was performed as follows: the subject sat facing the panel of numbers with his thumb and forefinger on a 1.25-in. control stick of a Grumann automatic pilot controller, model GR2. When the subject was ready, the experimenter threw the first switch, causing various pairs of numbers to flash off and on in an irregular manner. After approximately 10 seconds of this irregular flashing of the numbers, a second switch was thrown, activating the previously selected time delay relay and disconnecting the rotating switch. The panel of numbers now remained dark for the selected time period. This alerted the subject to be ready for the appearance of the two numbers previously selected by the experimenter. With the closing of the circuit lighting the selected pair of numbers, the Beckman time interval meter was activated. The subject at this time seeing a pair of numbers, had to decide whether the pair was even, odd, or an odd-even combination. For an even combination the stick switch was to be pressed to the right, for an odd combination to the left, and for an odd-even combination, forward. Moving the stick in the correct direction closed a microswitch which stopped the counter. If the wrong combination was chosen, the counter remained activated and the result was recorded as an error. The circuitry of this device is seen in Fig. 1.

It had been previously determined in an initial study of this apparatus that an individual's score for a given number pair was quite consistent, but that there were considerable differences in the times

between different pairs. For this reason, ten odd-even pairs in which the numbers were approximately the same distance apart on the panel had been tested repeatedly with a group of subjects. It was found that four of these pairs gave consistent reaction times varying between 825 and 875 msec. Each test consisted of five reaction times, two of which were obtained from the four consistent pairs. In the preliminary testing it was determined that the subjects were not aware of the fact that certain number pairs were being repeated.

Tracking Test.—The apparatus used to measure motor coordination was similar in principle to many previously employed and is shown in Fig. 2. The object of the test was to hold a 15 mm. spot of light from a pistol-like flashlight on a circular 1-cm. photocell that was moving at a distance of 2.5 ft. from the end of the light gun. The photocell unit consisted of a basic motor which moved a 7-in. arm through a vertical 70° arc, requiring 20 seconds for a complete cycle. To the end of this arm was connected a 15 r.p.m. synchronous motor, which in turn continuously rotated a second arm 6 in. in length, also in the vertical plane. At the end of the 6-in. arm was a 2-in. white circular target with a 1-cm. circular photocell in the center. The combined movements of both motors caused the photocell to travel at an irregular speed and in a varying pattern. The speed with which the photocell moved relative to the subject depended upon the relative motion of the component motors. When the directions of movement were the same, the photocell would move in a gradually accelerating or sudden burst of speed, or if moving in opposite directions, the photocell might almost stop or move very slowly. In order to induce irregularity further and prevent smooth continuous tracking, a universal joint was used to connect the 15 r.p.m. motor to the 6-in. target arm. By adjustment of this joint it was possible to cause the target to make a bobbing movement at four points with each circular motion of the 15 r.p.m. motor. As these irregular movements occurred at varying positions of the photocell and were dependent upon the relative positions of the two arms, the bobbing could not be anticipated. The entire apparatus

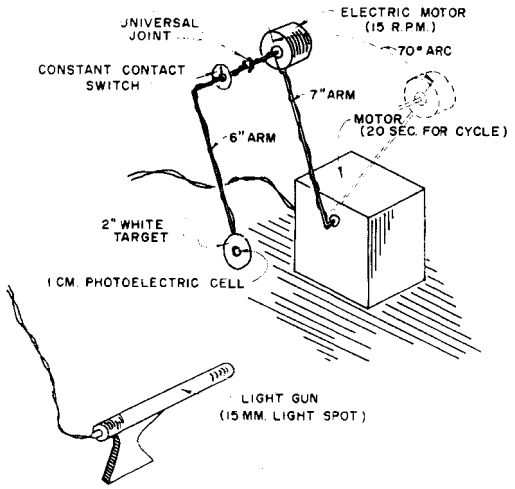


Fig. 2.—Diagram of mechanical portion of the tracking test apparatus.

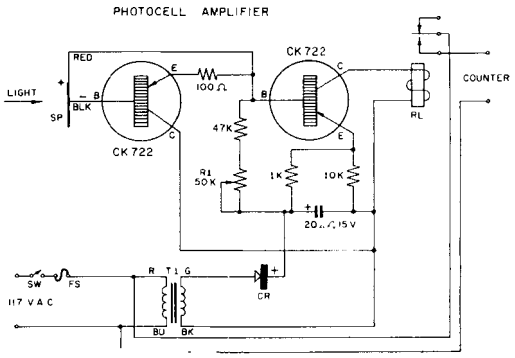


Fig. 3.—Circuit diagram of amplifier used to control the sensitivity of the tracking test photocell.

was placed in the rear of a blackened box with the open end to the front. The dimensions of the box were 26 in. high by 26 in. wide and 34 in. deep. A black screen with a slot through which the blackened rotating arm extended concealed the apparatus from the subject who saw only an erratically moving 2-inch white circular target with a 1-cm. black center.

The photocell was electrically connected through a constant contact rotating switch on the 6-in. arm to an amplifier relay circuit shown in Fig. 3. Adequate light upon the photocell induced a signal which, when sufficiently amplified, activated a relay and, in turn, closed a 60-cycle, 110 v. circuit connected to the Beckman time interval meter. Therefore, every second that the photocell was adequately illuminated, 60 counts would be recorded.

The experiment was performed with the subject sitting squarely before the target box having both feet flat on the floor. At a signal from the subject who was tracking the target, the experimenter threw a switch which simultaneously connected the Beckman time interval meter to the photocell circuit and started a 40-second timer. A perfect score for continuous contact throughout the 40-second period was a count of 2400. However, in order to allow for drug-induced increments or decrements in performance, the amplification of the photocell output was so adjusted that the average control score was less than half the perfect score. This test was repeated four times during each period.

Central Depressant Study

The study to determine the effect of central depressants upon performance was also conducted with 15 new subjects, but with the same experimental design and tests described for the stimulants. The drugs employed and their respective doses were: placebo, three doses; phenobarbital (as a standard sedative), 15, 30, and 45 mg.; chlorpromazine, 25, 50, and 100 mg.; meprobamate, 200, 400, and 800 mg.; and oxanamide, 200, 400, and 800 mg.

Central Stimulants Fatigue Study

This study was designed to elaborate further upon certain results obtained with the initial central stimulant experiments. One objective was to determine the effects of phenmetrazine and methyl phenidyl acetate upon muscular fatigue. Each of these drugs had appeared promising in the 60-second tapping test and no indication was found in the literature that they had been tested for this effect.

A second objective was to answer a hitherto unexplained question of how the central stimulants affect fatigue. The question was whether psychomotor drugs act to maintain the base-line level of performance by suppressing the sense of fatigue or whether, by actually raising the initial level of performance, they simply negate a fatigue-induced decrement. Two tests were conducted to determine these objectives. As the rate of tapping had been previously noted to decline steadily throughout the period of 60-second tapping, this test was extended to 3 minutes. Readings of the counts were made at half-minute intervals. The second test employed the tracking apparatus and was performed by extending the period of testing to 6 minutes, during which time readings were made at minute intervals. To induce fatigue further, the subjects were required to hold their arm with the light gun straight before them rather than with the elbow bent and touching the side.

Five subjects participated in this study. The treatments employed were: placebo, methyl phenidyl acetate 15 mg., and phenmetrazine 50 mg. Subjects received each treatment twice in randomized allocation and according to the manner previously described.

Chronic Deanol Study

Eighteen volunteer, male, college students between the ages of 21 and 33, weighing between 135 and 205 pounds, served as subjects. Orientation and tests were the same as for the studies with central stimulants and depressants. Subjects were randomly divided into two groups with nine subjects in each group. Group 1 received a daily capsule containing a placebo during the first four weeks and a capsule identical in appearance containing 75 mg. of deanol (dimethylaminoethanol) for the next four weeks. Group 2 received these treatments in the reverse order. Double blind conditions were maintained throughout. The five tests were administered in the same manner as in the central stimulant and depressant studies except that the testing of each subject was repeated at regular weekly intervals from the onset of treatment. In addition to the usual tests, subjects were also examined for changes in mood and any other subjective responses. For the analysis of a possible effect upon mood, the adjective check list of Nowlis (4) was employed. This list consisted of 145 adjectives descriptive of mood, feelings, or states of mind. Each adjective could be categorized as being either positive (cheerful, self-confident, etc.) or negative (depressed, unsure, etc.) in character. At each test period the subject checked those adjectives on the list which definitely described his feelings, using a single check were applicable, a double check mark where the adjective strongly applied, and a question mark when there was some uncertainty. In addition to the adjective list, subjects were further asked to describe any change from normal in their mental or physiological functions, such as the presence of pain, amount of sleep required, or changes in appetite, libido, study habits, and similar effects.

RESULTS

Central Stimulant Study.—In order to compensate for normal diurnal variations and to increase the precision of the tests of significance, the means of 12,480 raw scores were first adjusted for regression upon the corresponding control values. These adjusted scores were examined by an analysis of variance (5). The results are shown in Table I. The principal experimental variables were drugs, doses, and times. In each test, drugs constituted a significant source of variation. This means simply that not only were the drugs employed capable of affecting performance, but also that the tests selected were adequate to detect these changes. The absence of significant variations in dose response, except for the 60-second tapping rate, would indicate

TABLE I.—ANALYSIS OF VARIANCE OF ADJUSTED[a] POSTTREATMENT SCORES OF CENTRAL STIMULANTS

Source of Variance	*df*	Mean Square	*F*	*P*
		Pursuit Test		
Drug	3	6,266	11.33	<0.01
Dose	2	1,385	2.50	
Time	2	9,921	17.94	<0.01
Drug × dose	6	12,565	22.72	<0.01
Drug × time	6	1,989	3.60	<0.05
Dose × time	4	372	0.67	
Drug × dose × time	12	553		
Total	36			
		Five-Second Tap		
Drug	3	0.5849	3.63	<0.05
Dose	2	0.4688	2.91	
Time	2	0.9747	6.04	<0.05
Drug × dose	6	0.9610	5.96	<0.01
Drug × time	6	0.2665	1.65	
Dose × time	4	0.0884	0.55	
Drug × dose × time	12	0.1613		
Total	36			
		Sixty-Second Tap		
Drug	3	63.4259	6.53	<0.01
Dose	2	301.2100	31.01	<0.01
Time	2	125.5413	12.93	<0.01
Drug × dose	6	41.9323	4.32	<0.05
Drug × time	6	6.5628	0.68	
Dose × time	4	12.9989	1.34	
Drug × dose × time	12	9.7120		
Total	36			
		Visual Reaction		
Drug	3	1,255,955	6.19	<0.01
Dose	2	758,429	3.74	
Time	2	21,562	0.11	
Drug × dose	6	687,697	3.39	<0.05
Drug × time	6	171,169	0.84	
Dose × time	4	235,488	1.16	
Drug × dose × time	12	202,746		
Total	36			
		Complex Reaction		
Drug	3	37,381,699	3.56	<0.01
Dose	2	34,867,787	3.32	
Time	2	3,810,247	0.36	
Drug × dose	6	85,377,907	8.13	<0.01
Drug × time	6	11,104,987	1.06	
Dose × time	4	15,545,967	1.48	
Drug × dose × time	12	10,504,450		
Total	36			

[a] Adjusted score is the difference between mean treatment score and mean control score.

TABLE II.—EFFECT OF CENTRAL STIMULANTS AS MEAN PER CENT DEVIATIONS FROM CONTROL[a]

Treatments	Tracking Test			Five-Second Tap			Tests and Trials[b] Sixty-Second Tap			Visual Reaction			Complex Reaction		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Placebo	7.12	9.51	10.36	3.30	−2.01	−0.80	2.01	−0.10	−2.91	−6.33	−4.16	−6.37	4.81	1.90	−3.14
Caffeine, 100 mg.	25.43[c]	27.80[d]	31.95[d]	3.01	4.12	5.81	2.17	4.11	3.90	−3.68	−2.61	−5.63	14.00	7.75	10.84
Caffeine, 200 mg.	20.74	19.72	20.58	2.16	2.84	2.79	5.49	8.87[d]	6.32[c]	−0.28	−2.46	−4.20	6.51	0.17	7.24
Caffeine, 300 mg.	5.06	6.10	6.84	1.88	1.17	0.63	5.44	4.78	5.40[c]	1.32	−0.70	0.27	−1.43	−0.42	−3.40
d-Amphetamine, 2.5 mg.	7.92	15.34	21.07	1.78	2.67	2.30	3.86	5.06	4.75[c]	1.62	0.50	0.33	6.11	−4.15	−0.55
d-Amphetamine, 5.0 mg.	11.03	16.36	18.63	0.37	3.30	−0.14	2.94	5.59	4.41	−1.55	−1.14	0.49	−2.90	0.14	1.96
d-Amphetamine, 10.0 mg.	0.82	10.88	17.08	2.21	4.36	3.53	4.87	8.40[c]	6.42[d]	−0.15	4.47	1.47	5.41	10.89	9.84
Phenmetrazine, 12.5 mg.	5.14	7.21	10.28	0.76	3.14	4.22	2.41	3.62	2.34	2.51	3.41	8.95[d]	9.13	8.18	−0.35
Phenmetrazine, 25.0 mg.	13.52	19.26	23.17	2.91	3.89	4.89	3.40	6.09	6.66[d]	−0.23	0.44	−2.45	6.22	11.54	4.37
Phenmetrazine, 50.0 mg.	20.61	26.04[d]	29.43[d]	5.43	6.74[c]	3.85	7.95	7.20[c]	7.59[d]	−0.08	1.92	2.46	−3.57	−8.86	−9.83
Methylphenidate, 5 mg.	6.45	15.09	21.26	0.74	3.52	−1.18	1.35	2.47	0.59	1.00	−1.15	0.16	−1.93	−3.87	3.34
Methylphenidate, 10 mg.	15.89	22.33	26.35	3.13	5.34[c]	2.13	1.65	5.92	3.87	−1.05	1.23	−2.71	−4.28	−3.32	0.38
Methylphenidate, 15 mg.	23.08[c]	27.25[d]	29.96[d]	4.04	5.57[c]	3.87	3.21	6.86	5.87[c]	2.57	1.31	3.88[c]	0.34	6.91	−5.75

[a] Controls are those test values obtained immediately preceding drug administration. [b] Trials 1, 2, and 3 are those test values obtained 1, 2, and 3 hours after drug administration. [c] Significantly different than placebo ($P < 0.05$). [d] Significantly different than placebo ($P < 0.01$).

that either the tests were not sufficiently sensitive to detect the overall effect of the dose as a variable or that the differences in the sizes of the dose were not adequate to demonstrate such an effect. This does not, of course, preclude the possibility that individual doses may induce significant variations from corresponding control values. The fact that time was significant for all tests except the reaction times tells that the length of time following drug administration also influenced the response.

In order to determine which drug-dose-time performances were significantly different from those of the placebo, the *t*-test of Dunnett (6) was applied to the differences between the mean test scores and control values for the drugs and placebo. These data are presented in Table II and in Figs. 4–13. From Table II it is apparent that the tracking test was the most sensitive gauge of stimulant drug activity and that the lowest dose of caffeine, 100 mg., and the highest doses of phenmetrazine and methyl phenidyl acetate, 50 mg. and 15 mg., respectively, were generally the most effective.

The numerical values employed in Table II and their graphic presentation in Figs. 4–13 demonstrate the discrete changes resulting from the drug-dose-time interaction. Although with such presentations the individual values and treatments may be observed to vary significantly from those of the corresponding placebo or control, total drug-dose effects are not easily compared. In order to simplify such a comparison, the time-effect was integrated by measuring the areas under the graph curves with a planimeter. These values, measured in square centimeters, were obtained from original graphs in which the coordinates measured 7.6 cm. and 15.3 cm. for the x and y axes, respectively. The values obtained are the algebraic sums of the areas above and below the zero or control level, and are presented in Table III.

The 5-second tapping test afforded an objective index of the speed of a simple repetitive muscular movement rather than a measure of precision. It is most apparent from Table III and from Figs. 4 and 5 that methyl phenidyl acetate and phenmetrazine can significantly improve 5-second tapping. As might be anticipated, peak activities appeared at the second hour posttreatment. While it is not

Fig. 4.—Five-second tapping rate.

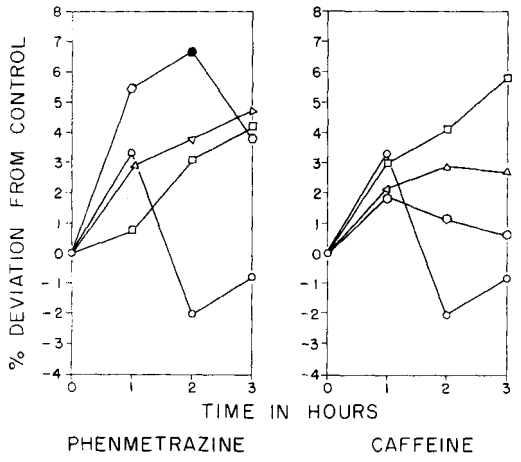


Fig. 5.—Five-second tapping rate.

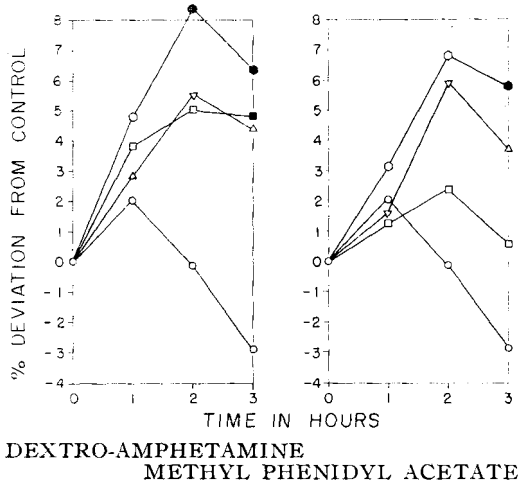


Fig. 6.—Sixty-second tapping rate.

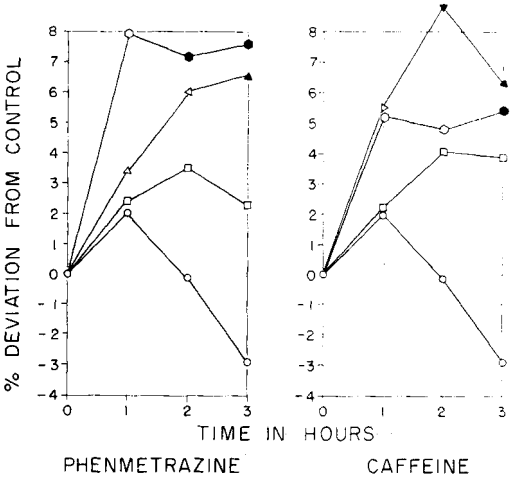


Fig. 7.—Sixty-second tapping rate.

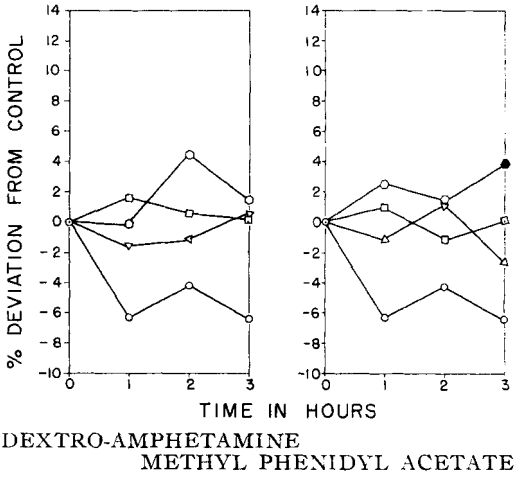


Fig. 8.—Simple visual reaction time.

surprising to find that central stimulants are capable of improving this type of performance, it is rather surprising that *d*-amphetamine did not cause a significant improvement. From Fig. 4 and Table II, it can be observed that *d*-amphetamine, while raising the level of performance over that of the placebo, was not as effective as the larger doses of phenmetrazine or of methyl phenidyl acetate. Further, these latter agents induce a dose-related effect. These differences may be the result of an improper selection of doses or they may reflect real differences in the actions of these drugs. The results with caffeine, although not significant, are most interesting because of the inverse relationship between dose and effect.

The information obtained from the 60-second tapping test is quite similar to that from the 5-second test, although the longer test appears to be a somewhat more sensitive measure of the drug effect. The principal difference in these two was the factor of fatigue in the longer test. All drugs demonstrated a significant improvement in performance at one or more of the times tested (Table II). From

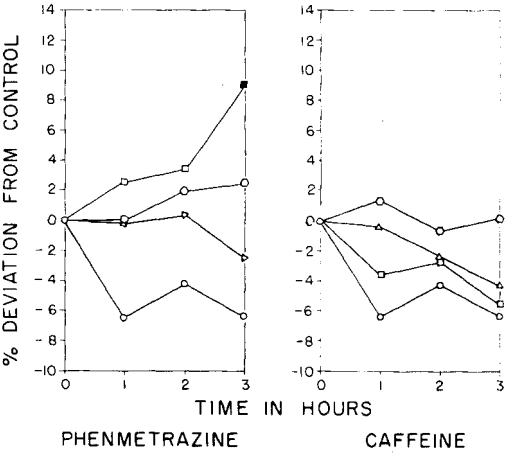


Fig. 9.—Simple visual reaction time.

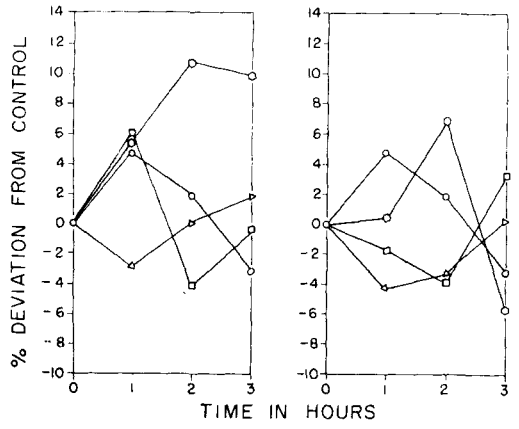


Fig. 10.—Complex reaction time.

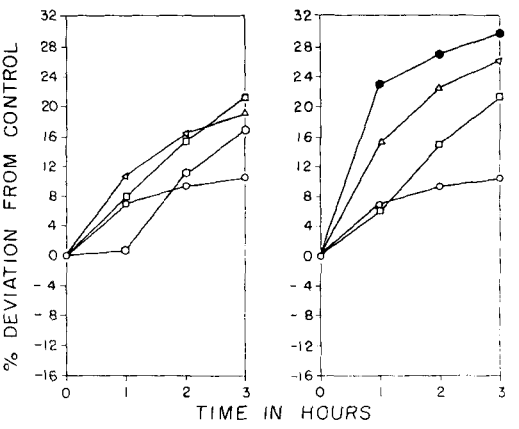


Fig. 12.—Tracking test.

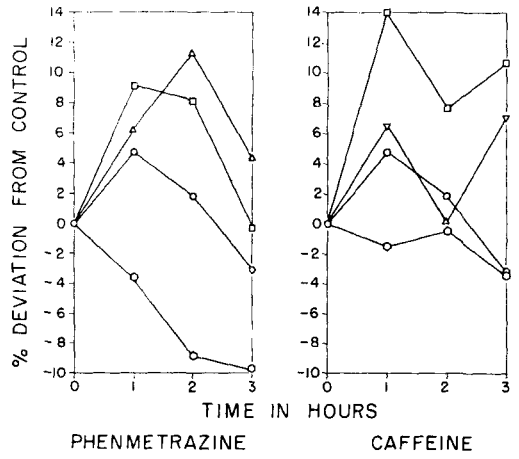


Fig. 11.—Complex reaction time.

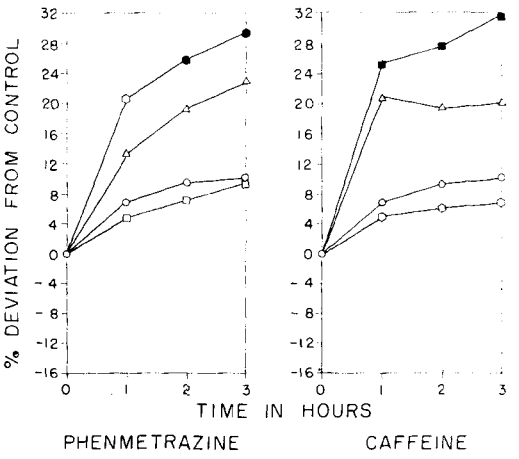


Fig. 13.—Tracking test.

Figs. 4—13.—Placebo, ○; *d*-amphetamine, □, 2.5 mg., △ 5 mg., ⬡ 10 mg.; methylphenidyl acetate; ⊏ 5 mg., △ 10 mg., ⬡ 15 mg.; phenmetrazine, □ 12.5 mg., △ 25 mg., ⬡ 50 mg.; caffeine, ■ 100 mg., △ 200 mg., ⬡ 300 mg. Filled-in symbols indicate a significant difference ($P < 0.05$) from corresponding placebo values.

TABLE III.—INTEGRATED DOSE-TIME EFFECTS OF CENTRAL STIMULANTS[a]

Treatments	Tracking Test	Five-Second Tap	Sixty-Second Tap	Visual Reaction	Complex Reaction
Placebo	17.9	2.0	1.9	−21.4	6.7
Caffeine, 100 mg.	56.0	32.5	27.6	−14.4	44.2
Caffeine, 200 mg.	41.1	21.0	55.5	−8.2	16.7
Caffeine, 300 mg.	12.2	12.6	40.9	1.0	−6.3
d-Amphetamine, 2.5 mg.	27.4	18.2	35.4	3.7	3.1
d-Amphetamine, 5.0 mg.	29.7	15.8	33.3	−9.9	−2.6
d-Amphetamine, 10.0 mg.	16.5	22.1	52.1	10.4	34.4
Phenmetrazine, 12.5 mg.	13.1	19.3	24.6	17.8	29.0
Phenmetrazine, 25.0 mg.	35.2	30.1	40.3	−2.5	31.9
Phenmetrazine, 50.0 mg.	49.2	45.8	60.4	5.4	−27.8
Methyl phenidyl acetate, 5.0 mg.	26.1	10.4	12.9	−0.3	−7.0
Methyl phenidyl acetate, 10.0 mg.	41.2	30.9	31.6	−1.7	−13.0
Methyl phenidyl acetate, 15.0 mg.	52.2	38.7	43.2	9.6	6.1

[a] The areas under curves were measured with a planimeter from original graphs in which the coordinates measured 7.6 cm. and 15.3 cm. for the *x* and *y* axes, respectively. Values are the algebraic sums of the areas above and below the zero or control level.

Table III and Figs. 6 and 7, it may be observed that the improvement was dose-related except in the case of caffeine. For reasons that are not apparent, the order of activity with caffeine was 100 < 300 < 200 mg.

The effects of the drugs upon both simple and complex reaction times were not as marked nor as consistent as those obtained with the tapping tests. This may be seen from examination of Tables II and III and Figs. 8–11. Generally some improvement over the control values can be noted, but of greater meaning is the fact that the time-related performance decrement of both tests, as seen by comparison with the placebo values, was prevented by most of the drug doses. Several effects stand out. First, in contrast to most of the other tests, in the simple reaction time, caffeine induced a slight improvement that was directly related to the dose. With the complex reaction time, however, the 100-mg. dose of caffeine produced significantly greater improvement than the higher doses, again demonstrating an inverse relationship between the dose and the effect of this drug. This effect of a high dose of a stimulant causing a decrease in the complex reaction time performance was marked with the 50-mg. dose of phenmetrazine.

From Tables II and III and Figs. 12 and 13, it is apparent that the tracking test was the most sensitive gauge of stimulant drug activity. Both methyl phenidyl acetate and phenmetrazine produced improvement in performance. In each case the effect was directly related to the size of the dose, and with the highest doses it was significant. Caffeine, on the other hand, once again demonstrated an inverse relationship between dose and effect with the 100-mg. dose inducing significant improvement. *d*-Amphetamine did not significantly affect this performance test at any dose nor at any time tested.

The side-effects observed with the central stimulants were minimal and usually consisted of no more than a marked increase in excitability and physical activity with the highest dose of each drug. All subjects were normotensive and no symptoms were elicited from the cardiovascular system other than moderate increases in the heart rate. One subject, who did not drink coffee, became quite agitated after the first dose of 100 mg. of caffeine and was given no further doses of this drug. Generally the subjective response to all the drugs was a feeling of well being, although the 300-mg. dose of caffeine had a tendency to induce nervous tension that might be best described as the "jitters."

Central Depressant Study.—Dunnett's *t*-test was applied to the data from the study of the central depressants in the same manner as previously described for the stimulants. The results are summarized in Table IV. Inspection of the mean values reveals that only one score was significantly improved. This was the simple reaction time with chlorpromazine 50 mg., at the 2-hour test period. Examination of the raw data from which this significant value was obtained gave no indication as to the reason for the apparent improvement.

While the values obtained in the various tests are seemingly quite variable in terms of the placebo effects, the per cent deviation from the control (initial) values are uniformly low (Table IV) in comparison with the results from the same tests using central stimulants (Table II). In the stimu-

Table IV.—Effect of Central Depressants as Mean Per Cent Deviation from Control[a]

Treatments	Tracking Test			Five-Second Tap			Tests and Trials[b] Sixty-Second Tap			Visual Reaction			Complex Reaction		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Placebo	3.24	6.73	5.42	1.46	0.68	0.85	0.53	0.93	1.33	−2.42	−4.78	−5.83	3.04	2.32	0.11
Chlorpromazine, 25 mg.	6.80	5.23	5.36	0.16	−2.72	−4.25	2.00	1.88	1.73	−2.85	−6.61	−6.61	10.24	3.41	8.22
Chlorpromazine, 50 mg.	0.88	1.89	−0.13	0.88	−0.94	−1.66	−0.57	−1.31	−1.88	3.21	6.10	3.83[c]	1.42	−1.23	−5.02
Chlorpromazine, 100 mg.	5.30	−0.25	−3.66	0.50	−2.20	−7.10	0.40	−2.07	−3.77	0.73	−3.88	−6.40	−14.31	−4.72	−4.72
Meprobamate, 200 mg.	2.62	2.86	4.11	0.03	−1.27	−2.78	1.65	3.55	3.39	−3.19	−3.66	−4.60	−1.38	−6.60	−3.30
Meprobamate, *400 mg.*	10.27	9.73	9.33	−0.32	0.82	1.83	1.27	1.05	1.05	−0.09	−0.27	−4.93	0.64	5.50	−1.15
Meprobamate, 800 mg.	4.29	6.24	7.61	0.92	0.48	−0.35	0.46	0.34	2.82	−3.49	−1.10	−3.30	−0.96	−12.33	−11.23
Oxanamide, 200 mg.	2.55	4.98	5.11	4.43	0.98	0.03	0.72	1.02	−4.09	−7.72	−7.86	3.71	1.06	5.30	
Oxanamide, 400 mg.	8.13	5.21	5.46	−0.28	−0.60	−2.62	0.82	1.58	1.77	−1.56	0.96	−6.75	0.27	−2.46	−3.69
Oxanamide, 800 mg.	−1.10	−2.44	−1.71	1.42	−0.03	−2.24	−0.68	−2.29	−1.43	−1.54	−4.90	−5.37	−0.40	−3.44	−5.83
Phenobarbital, 15 mg.	7.06	5.36	8.10	5.88	3.47	6.51	3.18	1.85	0.62	−0.52	−4.15	−3.35	−1.05	8.04	−2.50
Phenobarbital, 30 mg.	1.32	4.21	4.47	0.23	−0.97	−0.84	0.63	0.51	0.24	−4.74	−3.30	−5.16	−9.08	−7.15	−7.98
Phenobarbital, 45 mg.	3.47	3.59	5.57	3.03	−0.47	1.23	0.51	1.74	1.50	−1.16	−0.98	−4.41	−0.14	8.05	3.83

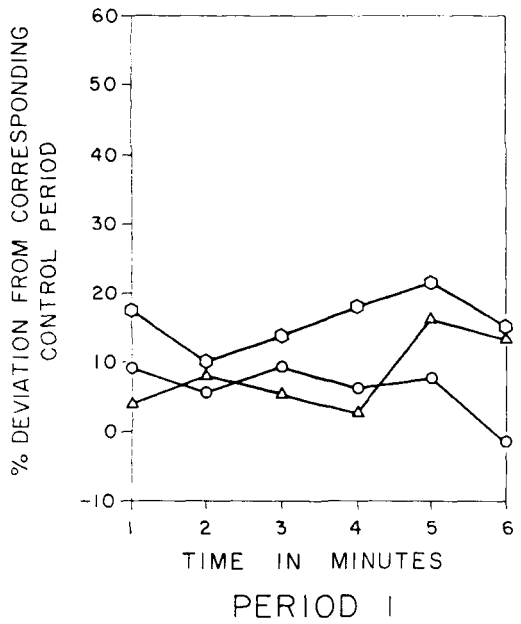
[a] Controls are those test values obtained immediately preceding drug administration. [b] Trials 1, 2, and 3 are those test values obtained 1, 2, and 3 hours after drug ad ministration. [c] Significantly different from placebo ($P < 0.05$).

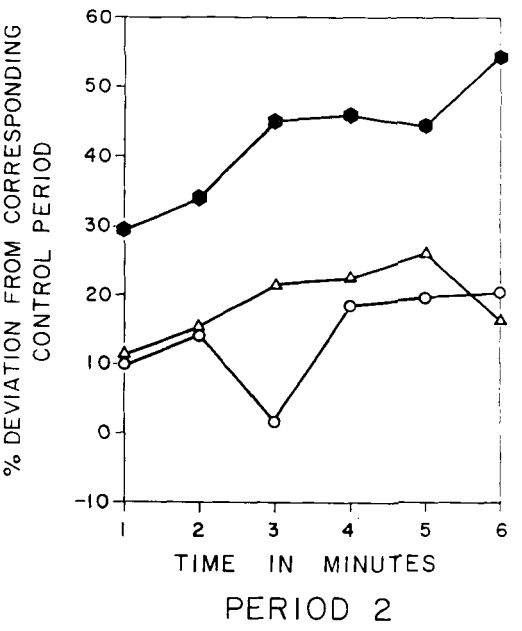
lant tests, the mean per cent deviation was frequently in the range of a 20 to 30% positive deviation, while with the central depressants, it seldom exceeded 8% in either direction.

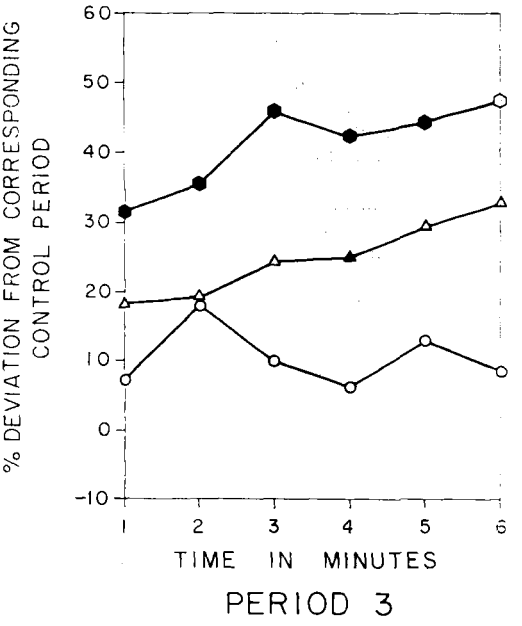
Central Stimulants Fatigue Study.—The results of this study are presented in Figs. 14–19. While the manner in which the data are presented does not indicate any apparent time-related decrement in performance, it should be noted that the points in each curve represent the per cent deviation from the corresponding points measured during the control period. Therefore, while the relative values shown in the curves may indicate an increase or little change in performance, a plot of the absolute values would result in curves having a definite downward trend in almost all cases. With this in mind, it may be seen that phenmetrazine induced a greater percentage improvement with the 6-minute tracking test than with the 3-minute tapping test. The prevention of fatigue with the stimulants, as anticipated from the results of the previous 60-second tapping test, was most prominent 2 hours after taking the drug. In the 6-minute tracking test the results were significant ($P < 0.01$) at all times throughout the 2-hour period and in all but one point at 3 hours. In the 3-minute tapping, only two points in the 2-hour test period were significant at $P < 0.01$ and $P < 0.05$, while none were significant at period three. Methyl phenidyl acetate significantly improved performance only at one time, 3 hours after the drug in the 6-minute tracking test.

Chronic Deanol Study.—The results of the performance tests were analyzed with a nested 2 × 2 factorial type design in order to measure the significance of each of the following effects: drug effect *vs.* placebo; order of administration, i.e., drug first, placebo last *vs.* placebo last, drug first; and finally the interaction between the drug and the period tested, i.e., drug-test period differences (7).

The mean values for the motor and psychomotor tests at 4 and 8 weeks are presented in Table V. In







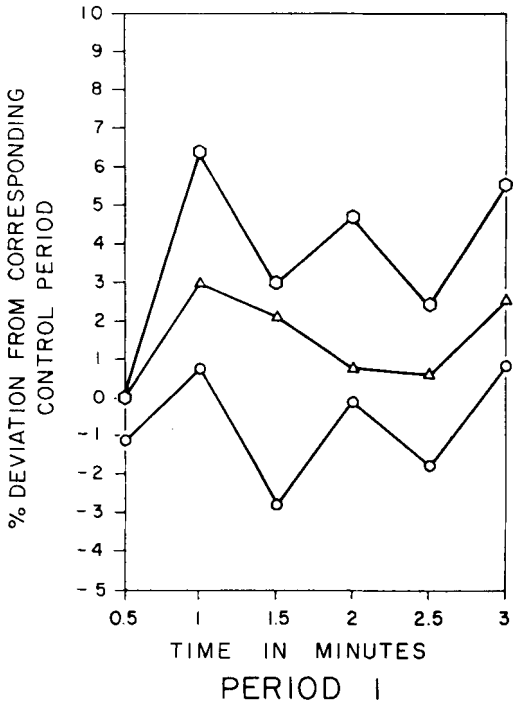
Figs. 14—16.—Six-minute tracking test; placebo, O; methyl phenidyl acetate 15 mg., △; and phenmetrazine 50 mg., ⬡.

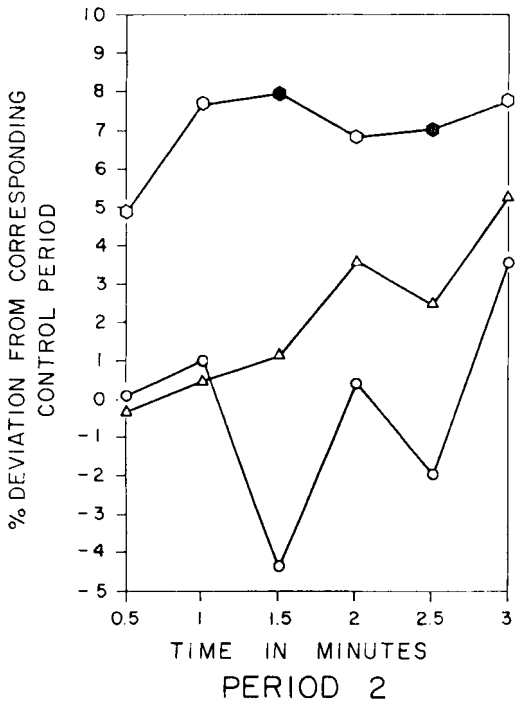
TABLE V.—MEAN CHRONIC DEANOL SCORES AT 4 AND 8-WEEK TEST PERIODS[a]

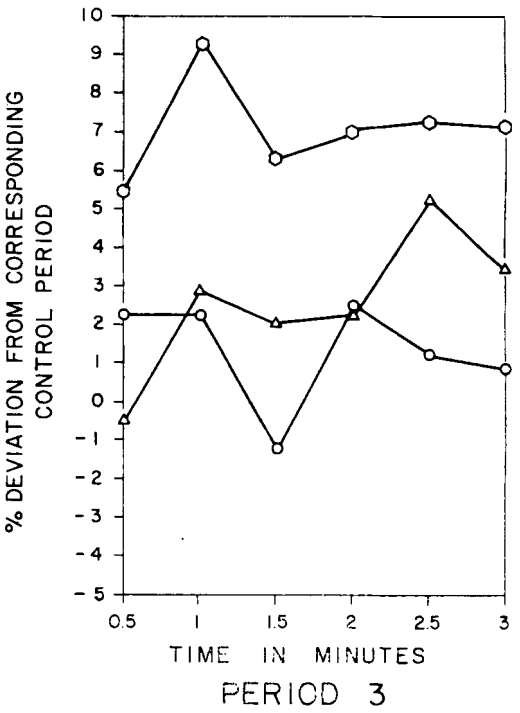
	Four Weeks		Eight Weeks	
	Placebo Group 1	Deanol Group 2	Placebo Group 2	Deanol Group 1
Tracking Test	660.2	720.7	649.8	635.2
5-Second tap	31.7	31.9	32.9	31.7
60-Second tap	326.0	333.4	329.1	321.2
Visual reaction	228.3	221.2	216.1	222.9
Complex reaction	814.2	764.0	728.3	733.2

[a] Group 1 received 75 mg./day of deanol for first 4 weeks while group 2 received a placebo. For the next 4 weeks, the treatments were reversed.

Table VI the analysis of variance of the data indicates a significant interaction only between order and drug for the pursuit test. It is apparent that the reason for the significance lies in the relatively high score obtained with the drug when it was administered in the first 4-week period. This score, when compared with the other three values in the pursuit test by a *t*-test, was found to be significant at $P < 0.001$ (8).







Figs. 17—19.—Three-minute tapping test, placebo, ○; methyl phenidyl acetate 15 mg., △; and phenmetrazine 50 mg., ⬡.

TABLE VI.—ANALYSIS OF VARIANCE OF CHRONIC DEANOL SCORES

Source of Variation	*df*	Mean Square	*F*	*P*
Tracking Test				
Order	1	12,656	0.96	...
Error term	16	13,233		
Drug	1	4,738	2.84	...
Drug × order	1	20,688	12.41	<0.01
Error term	16	1,667		
Five-Second Tap				
Order	1	4.41	0.12	...
Error term	16	36.51		
Drug	1	2.25	1.20	...
Drug × order	1	1.87	0.79	...
Error term	16	2.38		
Sixty-Second Tap				
Order	1	529.00	0.12	...
Error term	16	4,296.62		
Drug	1	0.44	...	...
Drug × order	1	186.78	0.99	...
Error term	16	189.42		
Visual Reaction				
Order	1	434.03	0.44	...
Error term	16	995.05		
Drug	1	0.25	...	...
Drug × order	1	250.70	1.03	...
Error term	16	243.03		
Complex Reaction				
Order	1	6,834	0.16	...
Error term	16	43,331		
Drug	1	4,624	0.34	...
Drug × order	1	30,625	2.28	...
Error term	16	13.458		

The results of the adjective check list test for mood were analyzed in several ways in order to obtain any possible indication of drug effect. As each adjective had a positive or negative mood characteristic, those that were checked were graded 3 points for a double check, 2 points for a single check and 1 point if they were undecided. The total number of positive points over the total negative points was expressed as the mood index. These values ranged from a low of 0.26 to a high of 79.0. Comparison of the 4 and 8-week values revealed no significant difference between drug and placebo. Neither was there any tendency for the mood index to increase during the 4 weeks of deanol administration when compared with the placebo administration. In some individuals, marked fluctuations in the mood occurred from week to week while with others, the values were relatively constant. Neither the direction nor the magnitude of the change could be related to the presence or absence of the drug nor to the period of testing. The comments of subjects concerning changes in mental or physiological functions were equally variable and unrelated to the drug or the period.

DISCUSSION

In any study of human behavior the selection of test procedures not only can influence the results but, unfortunately, is rather empirical. The general types of tests that may be employed can be categorized as: conditioned response, affect change, neurophysiological, perceptual, motor, mental, and perceptual-motor tests. Tests employed in these studies were selected in order to meet several criteria. Because of the large total number of individual drug-test scores (34,860) in addition to the pretest training, it was necessary to consider carefully the amount of time required for the actual performance of each test. In a more limited study, it might have been possible to select tests which would have been more sensitive to drug effects, although there is little information on this subject. Further requirements of each test were that the results must be reproducible, quantitative, and complement the other tests employed rather than measure essentially the same parameters. The simple visual reaction time met these requirements for reaction time tests. While the complex reaction time test employed was not a well-known procedure, it combined both simple mentation and reaction time, and it was thought that when compared with the results of the simple reaction time test it might serve to separate the complex reaction time into the time required for mentation and that needed to complete the simple reflex. Because of the variance within the complex reaction times, such a comparison was not feasible. The tapping tests provided objective indexes of motor activity in terms of the speed at which a simple voluntary movement could be repeated and the effect of fatigue upon an extended performance. The pursuit, perceptual-motor in nature, integrates visual perception with a complex motor response. It has been suggested that tests requiring a complex response are most sensitive to central drug effects (9, 10).

As caffeine was the first psychomotor drug to be known, a considerable number of performance tests have been conducted with this agent. Unfortunately, much of the evidence is conflicting. Its effect upon reaction time presents a case in point. In our study, caffeine induced a significant dose-related improvement in simple visual reaction time. It has, however, been reported to: decrease the reaction time (11–14), lengthen the reaction time (15, 16), produce no change in the simple reaction time after doses as large as 600–900 mg. of caffeine citrate (17–20), and to produce either a decrease or an increase dependent upon the size of the dose (21). Small doses between 60 and 240 mg. produced longer reaction times than the placebo, while larger amounts, 300–360 mg., shortened the times.

There are several possible reasons for these divergent results with such a seemingly simple test. In some of the investigations, cups of coffee were employed to measure the dose of caffeine, a technique which raises suspicion as to the actual amount of caffeine administered (13, 16). Further, the measurement of the reaction time (RT) is dependent upon numerous variables which makes the test fairly complex to administer. For one thing, it has been demonstrated that the reaction time is a function of the sense modality stimulated, auditory RT's being faster than visual RT's. Further, the number of sense organs stimulated affect the times, as any combination of auditory, visual, and electrical or shock stimuli induce faster RT's than do the individual stimuli. Additional factors influencing the RT include: stimulus duration and intensity, period of readiness, stress, age, body position, and the responding member (22). It is entirely possible that some of the reported effects may have been the result of uncontrolled variables. For example, the optimum period of readiness before the stimulus is presented has been determined to be between 1.0 and 4.0 seconds, a longer delay resulting in a lengthened RT. This was the approximate range employed in our study although, in some of the results cited, the authors have used indefinite periods of readiness or periods up to 10 seconds.

Perhaps the most significant finding with regard to caffeine was that in three of the five tests, that is 5-second tapping, complex reaction time, and the tracking tests, an inverse relationship was found to exist with regard to dose and effect. The only other drug which caused this effect was the 50-mg. dose of phenmetrazine in the complex reaction time. That increasing the dose of caffeine might be detrimental to performance has been previously reported for tracking tests (18, 22, 23). The doses employed in these observations were in the 200 to 300-mg. range. We observed that with a dose of 200 mg., performance was still improved and that it required 300 mg. to induce a modest and nonsignificant decrease in performance. Such minor differences could be related to subject or test variables.

The reason for the impairment of performance with a task such as tracking may be related to the fact that caffeine has been reported to reduce steadiness of the hand in doses of 300 mg. or more (13, 17, 18, 20, 21, 24–26). While there has been no mention of an improved steadiness with lower doses of caffeine, an inverse dose-effect relationship has been reported in a test of coordination in which the time required to thrust a stylus into a series of holes was measured. In this test, 60 to 120 mg. of caffeine improved the performance, while doses above 180 mg. slowed the response (21). This same

inverse effect that we also noted with the 5-second tapping test may help to explain reports that caffeine increases (17, 20, 21, 23), decreases (13), or produces no change (18, 27) in the tapping rate.

Obviously the effects of caffeine upon performance are dose-related and complex. It is suggested that the impaired steadiness or tremor induced by the high doses of caffeine may be the primary factor, or a direct extension thereof, in the tests in which the drug reduced performance, although it is difficult to equate muscle tremor directly with a test such as complex reaction time. Reasons for the improved performances with caffeine are presumably similar to those applying to the other psychomotor stimulants tested. It has been determined by others that caffeine heightens excitability, improves vigilance, facilitates simple intellectual tests, improves the mood, and decreases fatigue. Whether these are different expressions of the same effect is difficult to say, but any or all would likely have influenced the performance tests in this study. One may conclude that if caffeine is to be employed for the general improvement of performance, the dose should not exceed 100 mg. unless it can be definitely determined that the detrimental effect of larger doses is not a direct extension of the beneficial effect. In this case perhaps, the impairment can be negated through the concomitant administration of appropriate central depressants.

The results of the *d*-amphetamine testing were in general agreement with those previously reported. It had been found to induce somewhat equivocal results in the tapping rate. Both an increase (20) in the rate and no change (18, 28) have been reported. We observed a dose-related trend toward improvement which was not significant, although we did not use as high a dose as some of the reports which found improved performance. An important variable in this test is the state of fatigue. As indicated in the 60-second test, *d*-amphetamine produces a significant increase in the rate at both the 2 and 3-hour periods. Other workers have demonstrated that *d*-amphetamine can prolong the time that an individual can perform physically fatiguing work (29–32). Repeated testing, or testing with intervals longer than 5 seconds would be expected to increase further the minimal improvement which we noted in the 5-second test. While the *d*-amphetamine was not significantly effective in any test except 60 second tapping, which is a measure of fatigue, it has been reported that 5 mg. of the drug significantly improves the score in a multidimensional pursuit test (33). This difference may be attributed to the greater sensitivity of the multidimensional pursuit test.

Phenmetrazine and methyl phenidyl acetate significantly improved the performance in the 5 and 60-second tapping rate, visual reaction time, and the tracking test. It has been previously observed that subjects depressed with phenobarbital were able to perform simple arithmetic calculations faster when they received 20 mg. of methyl phenidyl acetate (34). Phenmetrazine was found to improve mental performance approximately 9% in well-rested subjects and 40% in the presence of fatigue (35). Studies comparable to those reported in this paper have not been disclosed, but within the limitations imposed by the drug doses and the tests employed, these agents gave indication of greater promise for the improvement of performance than did either caffeine or *d*-amphetamine.

The fact that the central depressants did not significantly affect the performance tests is not surprising. Low doses of central depressant drugs have been generally found to be without effect upon simple performance tests similar to the types which we used (36, 37). Kornetsky found that meprobamate did not affect learning or reaction times in a dose of 800 mg., but in a 1600-mg. dose, impairment of performance was observed (38). In general, tests requiring integration of several functions, such as a simulated automobile driving apparatus (8) or a multidimensional pursuit test (39) are sensitive to moderate central depression. While the statistical analysis of the data did not demonstrate any depressant drug effects, a less sophisticated approach of simply observing trends gives some indication of a depressed performance. For example, as seen in Table IV, the greatest reduction in performance with the complex reaction time occurred with the largest doses of the tranquilizers. Further, the two larger doses of chlorpromazine (50 and 100 mg.) produce a deteriorated performance in four out of the five tests. It had been reported that chlorpromazine at the 100-mg. level impairs the pursuit test, tapping rate, and hand steadiness (40).

The tranquilizers should not be necessarily considered as agents which reduce performance, as the ability to perform certain tasks may actually be improved in the presence of fear, anxiety, or tension. This action has been demonstrated with both oxanamide (41) and meprobamate (42) using subjects having demonstrable nervous tension. Unfortunately, the experimental production of anxiety or tension in human subjects has not been adequately studied. It has been demonstrated that with an increase in muscle tension, performance will improve, but beyond a certain amount of tension it will rapidly fall off (43). It was previously noted in the discussion of the central stimulants that increasing the dose of caffeine beyond 100 mg. generally resulted in a performance decrement. These larger doses of caffeine were also found to induce tremor and other signs of muscle tension. It is possible with high dosage levels of caffeine, and perhaps of other central stimulants as well, that the concomitant administration of a tranquilizer may improve the level of performance above that obtainable with a stimulant alone.

A number of investigators have suggested that both caffeine and the amphetamines prolong the period over which an individual can maintain the level of performance in physically fatiguing tests (29–31, 44–47). There has been little evidence, however, to indicate the exact mechanism by which fatigued performance is mitigated. These drugs may either maintain the base-line level of performance, thereby preventing a performance decrement, or they may elevate the initial performance level, thus allowing a decrement to occur without dropping below the base-line. Neither caffeine nor *d*-amphetamine were tested in this phase of the work as both phenmetrazine and methyl phenidyl acetate appeared to be equally or more active, resulted in a better dose-response relationship, and had not been previously tested for their effect upon fatigue. Phenmetrazine in a 50-mg. dose was significantly effective in both the 3-minute tapping and 6-minute

pursuit tests. From the initial points in Figs. 15, 16, 18, 19, it can be seen that the effect upon fatigue is accomplished in part by elevation of the initial level of performance and, as best seen in the tracking test curves, also by the time-related widening of the difference between the points for the placebo and phenmetrazine. This increase in difference reflects a reduction in the rate of fatigue-induced decrement.

From the results obtained with deanol, several possible conclusions may be reached. As there was no indication of activity with the mood test and most of the psychomotor tests, it may be concluded that in the dose and with the duration tested, deanol does not produce central stimulation of the magnitude observed with the other stimulants. The reason for the highly significant ($P < 0.001$) difference between the effect of deanol at the end of the first 4 weeks of the 8-week test period and at the end of the second 4 weeks, is not clear. If this difference is related to the drug, then there must have been an interaction between the drug and some unknown, uncontrolled factor that differed between the first and the last 4 weeks of testing. It is suggested that the factor might have been the heightened interest or motivation during the early stages of testing. Although this type of interaction has not been reported, it is of interest to note in Table V that both the drug and placebo scores in the pursuit test were highest at 4 weeks.

The observed effect of the deanol treatment upon mood and other subjective responses is at variance with reported results (48). The responses described by others: a more affable mood, greater ability to concentrate, the ability to stop smoking, increased tolerance of contact lenses, and less stimulation from amphetamines, were presumably obtained with low daily doses of from 10 to 30 mg. of the drug. Similar conclusions might be drawn from a casual or uncritical inspection of our own data. We found that nervousness was reported five times by individuals taking deanol and four times by the controls. An increased incidence of headaches was noted four times by the controls. An increased incidence of headaches was noted four times by deanol subjects and six by the placebo group. There were six reports that more sleep was needed with deanol and five with the placebo. Conversely, the need for less sleep was indicated nine times in the placebo group and eight times in the deanol subjects. An increase in libido was noted once in each group. It may be of some consequence that during the first 4 weeks both drug and placebo groups made a total of 96 comments concerning subjective responses, while during the last 4 weeks, only 54 comments were made. The suggestion, previously advanced, that the significant effect of deanol upon tracking performance during the first 4 weeks might be related to an interaction with a heightened initial interest, would tend to be supported by this evidence.

Any final interpretation of the experimental results, in terms of their possible application to useful tasks, must be predicated upon the specific tests which were employed. Because a particular drug could not be demonstrated to affect a specific test does not mean that activity might not be observed, perhaps, in a more appropriate or total measure of performance. Conversely, if a drug was demonstrated to be effective, it may generally be assumed that while more sensitive test procedures would also be affected, total performance may still not be improved.

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Analysis of Phenobarbital in Relation to its Degradation Products

By FREDERICK TISHLER†, LEE F. WORRELL‡, and JOSEPH E. SINSHEIMER

The effect of the degradation products of phenobarbital upon six of its quantitative literature assays has been studied. Two new colorimetric procedures and an infrared method for the determination of phenobarbital are described and the effects of degradation products upon these assays is also discussed. Differential ultraviolet spectrophotometric analysis was found to be the assay procedure most useful for the determination of phenobarbital in the presence of its degradation products. This analysis has also been successfully applied to phenobarbital in various dosage forms.

THE LITERATURE contains little information of the effects of the degradation products of barbiturates upon the quantitative assays of the respective barbiturates. Jackson, *et al.* (1), have shown that the decomposition products of barbital did not interfere with its ultraviolet spectrophotometric assay as described by Goldbaum (2).

Since the instability of barbiturates (3-5) and the isolation of their decomposition products (6, 7) are well described in the literature, it was the purpose of this investigation to study the effects of the hydrolysis products of phenobarbital upon its most widely used quantitative assays. The decomposition products of phenobarbital which were used in this study included the following: phenylethylacetylurea, phenylethylmalonic acid, phenylethylmalonamide, α-phenyl-*n*-butyric acid, α-phenyl-*n*-butyramide, and urea.

The quantitative methods chosen for the study included: (*a*) the U.S.P. gravimetric assay (8), (*b*) a volumetric precipitation titration (9), (*c*) a nonaqueous titration (10), (*d*) the cobalt colorimetric procedure (11), (*e*) a direct ultraviolet spectrophotometric method (12), and (*f*) a differential ultraviolet spectrophotometric method (2, 13, 14). In addition to the above literature procedures, two new colorimetric assays were developed and their applicability for the analysis of phenobarbital in the presence of its degradation products was studied.

EXPERIMENTAL

Apparatus and Materials

A Beckman model DU spectrophotometer and a Zeromatic pH meter, and a Perkin-Elmer model 21 infrared spectrophotometer were the instruments used in this investigation. The melting points reported are corrected.

Phenobarbital, U.S.P. grade, was recrystallized from dilute ethanol, m.p. 176-178°.

Phenylethylacetylurea was prepared by thermal degradation of a 5 per cent aqueous solution of sodium phenobarbital and isolation of the precipitate. Recrystallization from dilute ethanol gave a melting point of 147-149° (3, 6, 7).

Phenylethylmalonic acid was prepared by the hydrolysis of the diethylester of the acid with potassium hydroxide according to the procedure of Tasselly, *et al.* (15). Recrystallization from benzene gave a melting point of 155-158° with liberation of carbon dioxide (6, 7).

Phenylethylmalonamide was prepared by heating the corresponding acid with phosphorus pentachloride on a steam bath for 6 hours. The phosphorus oxychloride formed was distilled off with the aid of a steam bath and water aspirator. The crude phenylethylmalonyl chloride was then added to a well cooled ether solution saturated with ammonia. The precipitated amide was dissolved in acetone and, after evaporation of the solvent, was recrystallized from dilute ethanol, m.p. 119-121° (6, 7).

α-Phenyl-*n*-butyric acid, m.p. 42-44°, and α-phenyl-*n*-butyramide, m.p. 83-84° were purchased from Eastman Organic Chemicals and recrystallized from dilute ethanol.

Urea, A.R., m.p. 132-133°, was used as purchased from Mallinckrodt.

Sörenson phosphate buffer, pH 7.38. The buffer is prepared by mixing 20 ml. of sodium acid phosphate solution (9.21 Gm. of $NaH_2PO_4 \cdot H_2O$/1000 ml.) and 80 ml. of dibasic sodium phosphate solution (9.47 Gm. of Na_2HPO_4/1000 ml.).

Elimination of Acidic Impurities

The following procedure was applied to mixtures of phenobarbital and its acidic degradation products, each of which was present in an amount equivalent to a 50% decomposition of 0.1 Gm. of phenobarbital: The mixture, after evaporation of any solvent, was mixed with three 10-ml. portions of petroleum ether (40-60°), in which phenobarbital is insoluble and α-phenyl-*n*-butyric acid is soluble. Each portion was decanted, filtered, and the petroleum ether solution discarded. The filter paper was washed with several portions of acetone and added to the petroleum ether-washed mixture. After evaporation of the solvent, 30 ml. of Sörenson phosphate

Received October 25, 1961, from the University of Michigan, College of Pharmacy, Ann Arbor.
Accepted for publication November 2, 1961.
Abstracted in part from a thesis presented to the University of Michigan Graduate School by Frederick Tishler in partial fulfillment of the requirements for the Ph.D. degree.
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buffer (pH 7.38) was added and the mixture stirred with a magnetic stirrer for one-half hour. Twenty milliliters of chloroform was added and the mixture stirred for an additional 5 minutes. The chloroform layer was drawn off by use of a separator and the aqueous phase was extracted with two additional 20-ml. portions of chloroform. The combined chloroform extract, after evaporation of the solvent, was then employed in the various analyses.

Assays

Differential Ultraviolet Analysis.—A sample of about 0.100 Gm. of phenobarbital is dissolved in an ammonia-ammonium chloride buffer (pH 10.0 ± 0.2) in a 1000-ml. volumetric flask. One 10-ml. portion of this solution is diluted with the buffer, pH 10.0, in a 100-ml. volumetric flask. A second 10-ml. sample is transferred to a 100-ml. volumetric flask, diluted to volume with buffer, pH 10.0, and 10 ml. of 1.0 *N* hydrochloric acid (final pH 1.5 ± 0.2). The absorbance of the alkaline solution is determined relative to the acid solution in the reference cell at 241 mμ. A blank solution was prepared and was found to have no contribution due to reagents and solvents at 241 mμ.

Dimethylglyoxime-Thiosemicarbazide Colorimetric Procedure.—A sample of phenobarbital, 0.005 Gm./10 ml. of 0.1 *N* sodium hydroxide, is decomposed at 100° for 90 minutes. Five milliliters of 1.0 *N* hydrochloric acid is added immediately to stop the degradation. A 1-ml. quantity of dimethylglyoxime solution (3% w/v in concentrated hydrochloric acid) and 1.0 ml. of thiosemicarbazide solution (1% w/v in 70% ethanol) are added. The mixture is heated for 105 min. at 100°. After cooling, the solution is diluted to volume in a 25-ml. volumetric flask with distilled water and the absorbance determined at 532 mμ relative to distilled water.

Copper-Pyridine Colorimetric Procedure.—The sample of phenobarbital, approximately 0.050 Gm., is dissolved in 4 ml. of a 4% ammonium hydroxide solution. To this solution is added 2 ml. of a copper-pyridine reagent (2 ml. of pyridine and 25 ml. of a 3% copper sulfate solution). After 3 hours, the solution is filtered with aid of a filtering stick and washed with several small portions of distilled water. To the suspension of the colored precipitate in distilled water, 5 ml. of concentrated ammonium hydroxide solution is added, and the precipitate decomposed in a steam bath. The solution is quantitatively transferred to a 500-ml. volumetric flask and diluted to volume with distilled water. The amount of copper present is determined colorimetrically with sodium diethyldithiocarbamate by the procedure of Charlot and Bezier (16) on a 2-ml. aliquot sample. The amount of copper is related in turn to the concentration of phenobarbital present.

Quantitative Infrared Analysis of Phenobarbital in Combination with Phenylethylacetylurea.—*Method I.*—The sample to be determined is dissoved in an amount of reagent chloroform so that the concentration of phenobarbital is 1% or less. The absorbances at 5.75 and 6.39 μ are determined from the spectrum produced in an 0.1-mm. cell with chloroform as the blank and the results compared to standards. The 5.75 μ band represents the concentration of phenobarbital and the 6.39 μ band represents the concentration of the ureide.

Method II.—The spectrum is obtained as directed under *Method I.* The amount of phenobarbital present is calculated from its 5.75 μ band. A blank sample is made containing this amount of phenobarbital in chloroform and the unknown sample is run against this blank. The concentration of ureide is determined from its 5.83 μ band.

DISCUSSION

The quantitative literature assays of phenobarbital were tested for interference using an amount of each degradation product corresponding to a complete hydrolysis of phenobarbital. Several of the decomposition products were found to interfere with the assays. The acidic decomposition products interfered with the gravimetric, nonaqueous titration, and cobalt colorimetric procedures. The use of a Sörenson phosphate buffer, as described above, was found to be the method of choice for the removal of acidic decomposition products. Rotondaro (17) has employed a bicarbonate purification procedure but in our hands, inconsistent recovery of the barbiturate was obtained due to the variation in pH of the bicarbonate solution.

The ultraviolet spectra for the degradation products of phenobarbital under the same conditions used by Mattson (12) for his determination of barbiturates are given in Fig. 1. At the wavelength of 240 mμ used in this analysis, the ureide would cause the greatest interference. Since differential ultraviolet spectrophotometric methods have been shown to be more selective for barbiturates, the

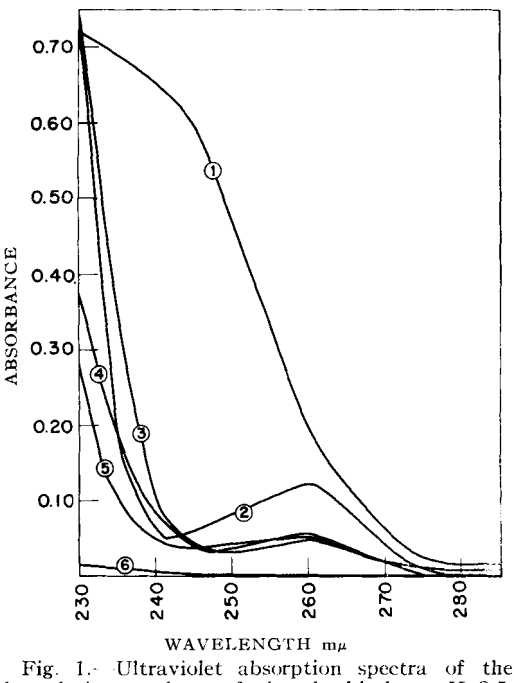


Fig. 1.—Ultraviolet absorption spectra of the degradation products of phenobarbital at pH 9.5. 1, Phenylethylacetylurea (0.1 mg./ml.); 2, α-phenyl-*n*-butyric acid (0.1 mg./ml.); 3, phenylethylmalonic acid (0.05 mg./ml.); 4, phenylethylmalonamide (0.05 mg./ml.); 5, α-phenyl-*n*-butyramide (0.05 mg./ml.); 6, urea (0.1 mg./ml.).

TABLE I.—COMPARISON OF ASSAYS

Technique	Sensitivity, (Sample Size)	Accuracy and Precision, Av. % Recovery ± S. D.	
Gravimetric	0.200–0.250 Gm.	98.1 ± 0.7[a]	...
Volumetric precipitation	0.100 Gm.	101.9 ± 1.0	102.1 ± 1.1[b]
Nonaqueous titration	0.100 Gm.	99.9 ± 0.1	99.9 ± 0.2[c]
Cobalt colorimetric	0.005 Gm./25 ml.	100.2 ± 1.5	98.3 ± 1.7[c]
Differential ultraviolet method	0.001 Gm./100 ml.	99.9 ± 0.1	99.9 ± 0.1[b]
Dimethylglyoxime-thiosemicarbazide method	0.005 Gm./25 ml.	99.2 ± 0.9	...
Copper-pyridine method	0.005 Gm./50 ml.	99.8 ± 1.0	99.7 ± 1.0[b]
Infrared analysis	0.125 Gm./25 ml.	...	97.8 ± 1.0[d]

[a] The results in this column are based on the analysis of pure samples of phenobarbital. [b] Determined in the presence of all the degradation products. [c] Determined after the elimination of interfering degradation products. [d] Determined in the presence of phenylethylacetylurea.

influence of the degradation products on this technique was studied.

Williams and Zak (13) and Goldbaum (2) have employed the difference maxima obtained from the spectra of the first and second ionic species of the barbiturate for quantitative purposes, while Walker, *et al.* (14), used the difference maxima obtained from the nonionized and first ionized species for the determination of barbiturates in urine and blood. Since the difference maxima involving the second ionic species depends on the use of extremely basic and, therefore, unstable solutions of phenobarbital, this procedure was not employed. The method of Walker, *et al.*, was further investigated and modified as described in the experimental procedure. The absorbance of each of the degradation products at 241 mμ was found to be constant between the pH range of 1 to 12. Thus, the degradation products do not interfere with this differential method of analysis.

In addition to the literature assays, two new colorimetric procedures have been developed and their usefulness for the assay of phenobarbital in the presence of its degradation products was investigated. The dimethylglyoxime-thiosemicarbazide procedure is based upon a qualitative test of urea compounds as described by Ohkuma (18). Since this method depends on the decomposition of phenobarbital with the formation of urea and phenylethylacetylurea, a sample should be tested prior to hydrolysis for a positive reaction due to these degradation products. The other decomposition products do not interfere with the analysis. At the end of 1 hour, the blank used in this determination becomes cloudy. However, since the absorbance due to the blank is negligible at 532 mμ, distilled water is employed as the blank.

Copper-pyridine derivatives of barbiturates have been investigated by Zwikker (19), Fouchet (20), and Levi and Hubley (21). The formation of this phenobarbital derivative followed by the quantitative determination of its copper content was found to be useful for the determination of phenobarbital in combination with its degradation products.

Infrared spectroscopy was found to be a useful procedure in the qualitative and quantitative determination of phenobarbital both alone and in the presence of its main degradation product phenylethylacetylurea. Phenobarbital exhibits a carbonyl doublet which serves as a criterion of the purity of phenobarbital. When the ratio of the 5.75 and 5.89 μ carbonyl doublet for phenobarbital is not 1 to 1.26, the presence of ureide is indicated. Method I or method II can be used to determine the concentration of phenobarbital and phenylethylacetylurea. Method II should be employed when the concentration of the ureide is less than 0.25% since the 6.39 μ band of the ureide does not follow Beer's law below this concentration. A comparison of the methods investigated is summarized in Table I. The nonaqueous titration and the differential ultraviolet method are highly satisfactory with regard to accuracy, precision, and efficiency. Where degradation products are present, the differential ultraviolet method offers the additional advantage of eliminating a prior separation of interfering products. The differential ultraviolet procedure has been used in these laboratories in the kinetic study of the degradation of phenobarbital (22).

TABLE II.—DIFFERENTIAL ULTRAVIOLET ANALYSIS OF PHENOBARBITAL ELIXIR U.S.P.

Amount Present		4.00 Gm./L.
Found	1	3.98
	2	3.99
	3	3.98
	4	3.99
	5	4.00
Average		3.99 Gm./L.

To serve as a basis for the quantitative determination of phenobarbital preparations, the difference in molecular extinction coefficient, $\Delta\epsilon$, was determined from standard solutions. The average of 10 determinations gave a value of 9404 for a molar solution of phenobarbital at a pH of 10 ± 0.2 over that of a molar solution of a pH of 1.5 ± 0.2 at 241 mμ. To test the applicability of the differential analysis to a pharmaceutical formulation, phenobarbital elixir U.S.P. (8) was prepared and analyzed for its phenobarbital content. In Table II are presented the results of the analysis. No interference of the components of the elixir was found in the differential ultraviolet analysis, while Mattson (12) reported that the amaranth interfered with the direct ultraviolet procedure and an elixir base was necessary as a blank.

Ephedrine and amphetamine do not show changes in ultraviolet spectra under the conditions of the differential ultraviolet method, and therefore the general usefulness of this method is further illustrated in the analysis of phenobarbital combinations of the above compounds.

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Pharmaceutical Investigation of Selected Alberta Bentonites I

Geology and Identification

By ARTHUR J. ANDERSON† and ELMER M. PLEIN

Bentonite samples were collected from a number of Alberta deposits. Of these, 23 were indexed in table form to show location and other information dealing with the parent beds. On a selected suite of samples, identification as bentonites was confirmed by differential thermal and X-ray diffraction techniques.

EARLY INFORMATION concerning bentonite deposits in western Canada for the most part is found as brief reports in publications of the Canadian government (1–4). Of particular value in this respect are the comprehensive surveys of Spence (5) and Spence and Light (6).

In 1949 the discovery of major oil reserves in Alberta brought an unprecedented demand for the high-swelling type of clay. With no known reserves of suitable quality in Canada, all supplies, of necessity, were imported from the United States. In recent years, however, intensive subsurface exploration programs carried out by commercial interests resulted in the location of a number of reserves considerably better in quality than those previously described. Of these, deposits uncovered at Rosalind and Onoway were considered sufficiently important to warrant commercial development. Mines and processing plants were established at both locations with production designed to meet the needs of the petroleum and associated industries.

Our interest in the material arose when claims were made that certain beds contained bentonite comparable in some respects to that obtained from the Black Hills region of Wyoming and South Dakota. A subsequent inquiry revealed that insufficient information was available to allow for an adequate pharmaceutical evaluation of bentonites from any of the known Alberta deposits. For that reason, a study was undertaken with a view to determining the presence of reserves of pharmaceutical grade clay in the province.

GEOLOGY

Byrne (7) states that in Alberta, thin beds of bentonite are fairly common throughout the Cretaceous and Tertiary eras. However, thick accumulations have been reported only from the upper Cretaceous, with the Edmonton formation undoubtedly being the most important for future prospecting (Fig. 1). The occurrences found in other levels of the upper Cretaceous as well as in the Paskapoo member of the Tertiary are considered to have little commercial importance.

Received August 21, 1961, from the College of Pharmacy, University of Washington, Seattle 5.
Accepted for publication October 11, 1961.
Based on a thesis presented to the Graduate School of the University of Washington by Arthur J. Anderson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
This research was supported in part by the Canadian Foundation for the Advancement of Pharmacy.
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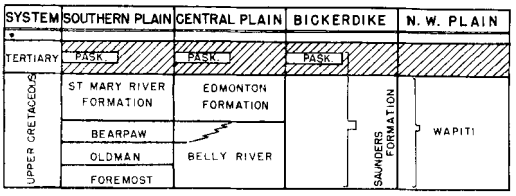


Fig. 1.—Stratigraph table of bentonite-containing formations in Alberta. *, Quaternary; Pask., Paskapoo.

In general, the Edmonton formation consists of poorly indurated shales and clays mixed with feldspathic siltstones and sandstones. Beds of relatively pure bentonite usually are found as lenses of varying widths. Coal seams are plentiful, and iron concretions are sometimes encountered.

EXPERIMENTAL

Collection and Preparation of Samples.—Since bentonite beds are known to vary widely in properties within short distances, a number of core samples are normally taken from each deposit to obtain an appraisal of clay quality throughout the occurrence. In this study, such a program was considered impractical because of transportation difficulties and the limited facilities available. Instead, every effort was made to obtain clean material, at varying depths below the surface of the deposit. In some instances a trenching procedure was employed to prevent contamination with surface sand. Several portions were gathered from different parts of each deposit and mixed to form a composite.

It was recognized that such samples were still not truly representative. For this reason, subsequent laboratory examination was considered to give only a general indication of the quality of the parent beds.

Twenty-nine samples were examined in the course of this work (Table I). Clays collected during field trips averaged between 8 and 10 lb. in weight, while samples received from other sources varied widely in size from 200 Gm. to 9 Kg. Coarse contaminants were removed by hand in the laboratory and the remaining material was dried in an oven at 90°. Each sample was reduced to a fine powder (200 mesh), mixed well to obtain a uniform blend, and then stored in a suitable glass, metal, or polythene container.

Identification of Clays.—Even though most of the samples used in this study were obtained from bentonite deposits which previously had been described by professional geologists, all clays were again identified as bentonite by qualified mineralogists either during or after collection. Recognized field tests were employed for this purpose.

After laboratory investigation had been conducted for some time it was felt that instrumental identification of the better and more interesting samples would be desirable. Arrangements were then made to have a suite of selected clays subjected to analysis by differential thermal and X-ray diffraction techniques.

Differential Thermal Analysis.—In recording the thermograms of the clay samples, a heating rate of 15° per min. was maintained over the entire temperature range (Figs. 2 and 3). Arrangement of the thermocouples, design of the block, and other details of the equipment used are described by Berkowitz (8).

TABLE I.—INDEX OF BENTONITE SAMPLES STUDIED

Sample[a]	Geologic Formation	Location	Map Coordinates
1	Edmonton	Rosalind[b]	Lsd. 15, Sec. 31, Tsp. 42, R. 17, W. 4
1A	Edmonton	Rosalind	Lsd. 15, Sec. 31, Tsp. 42, R. 17, W. 4
2	Edmonton	Rosalind	Lsd. 15, Sec. 31, Tsp. 42, R. 17, W. 4
3	Edmonton	Rosalind	Lsd. 15, Sec. 31, Tsp. 42, R. 17, W. 4
4	Edmonton	Drumheller	N.W. 1/4, Sec. 14, Tsp. 29, R. 20, W. 4
5	Edmonton	Beynon	S.E. 1/4, Sec. 32, Tsp. 27, R. 20, W. 4
6	Bearpaw	Dorothy	N.E. Sec. 3, Tsp. 27, R. 17, W. 4
7	Bearpaw	Irvine	N.W. 1/4, Sec. 30, Tsp. 11, R. 2, W. 4
8	Upper Oldman	Walsh	S.W. Sec. 28, Tsp. 11, R. 1, W. 4
9	Bearpaw	Bullshead Butte	N.E. 1/4, Sec. 2, Tsp. 8, R. 7, W. 4
10	Edmonton	Busby	N.E. 1/4, Sec. 7, Tsp. 56, R. 2, W. 4
16	Edmonton	Nevis	S.W. 1/4, Sec. 22, Tsp. 39, R. 22, W. 4
20[c]	...	...	Volclay, B.C. Grade.
21	Edmonton	Rosalind	
22	Edmonton	Rosalind	
23	Edmonton	Smoky River[d]	Lsd. 6, Sec. 35, Tsp. 69, R. 4, W. 6
24	Wapiti	Kleskun Hills	Sec. 15, Tsp. 72, R. 4, W. 6
25	Wapiti	Kleskun Hills	North Edge Secs. 21, 22, 23, Tsp. 72, R. 4 W. 6
26	Wapiti	Kleskun Hills	North Edge Secs. 21, 22, 23, Tsp. 72, R. 4, W. 6
27	Edmonton	Smoky River[d]	Lsd. 4, Sec. 2, Tsp. 70, R. 3, W. 6
28	Edmonton	Rosalind	
29	Control		Volclay, B.C. Grade
30	Edmonton	Onoway	Lsd. 9, Sec. 7, Tsp. 56, R. 2, W. 5
31	Edmonton	Onoway	Lsd. 9, Sec. 7, Tsp, 56, R. 2, W. 5
32	Edmonton	Rosalind[e]	

[a] Samples not listed were rejected after preliminary examination. [b] Rosalind beds contain several grades of clay roughly classified on a color basis: green (1, 1A, 21, 22, 28), gray (2), and yellow (3). [c] A small sample of finely ground bentonite received from the American Colloid Co. for testing. When it proved satisfactory for use as a control, a larger quantity was supplied for this purpose (sample 29). [d] Not previously reported. [e] Fines collected from vent stack of Rosalind mill.

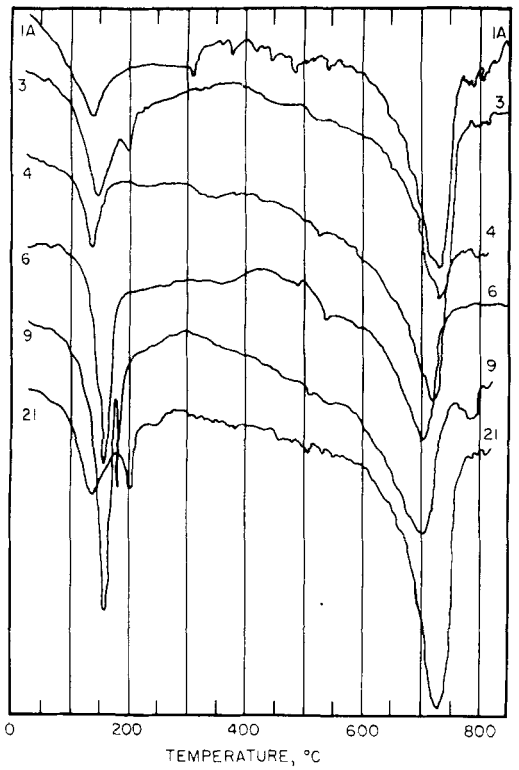


Fig. 2.—Differential thermal analysis of selected Alberta bentonites.

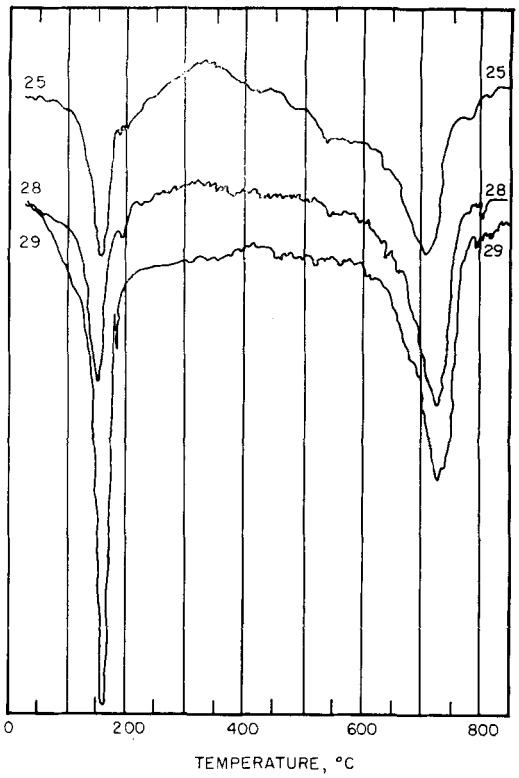


Fig. 3.—Differential thermal analysis of selected Alberta bentonites.

X-Ray Diffraction Analysis.—The clay portion of bentonite has been shown to consist primarily of montmorillonite. With the discovery that this material was actually crystalline in nature, it became possible to employ X-ray techniques to measure the characteristic angles of reflection of such rays from the atomic planes of the crystals.

A 2% (w/w) suspension of clay was prepared in a Waring Blendor (9). This mixture was transferred to a measuring cylinder and allowed to stand undisturbed for 8 hours and 10 min. By means of a pipet, 25 ml. of the suspension was then withdrawn from immediately below the surface and allowed to run into a clean Petri dish containing two glass microscope slides which were completely covered in the process. The assembly was then dried in an oven at 55–60°, and the slides removed with the aid of a razor blade to prevent chipping the oriented clay film. The prepared sample was then placed in a Phillips X-ray spectrometer and exposed to a monochromatic beam obtained through the use of a copper target and nickel filter. Scanning rate was set at 1° per min., through 40°, with the tracing recorded on a synchronized Brown strip chart recorder.

A diffraction tracing of the clay film was obtained, after which the slide was transferred to a glass desiccator and exposed to ethylene glycol vapor for 1 hour at room temperature. A second tracing was then prepared as before.

Basal (001) spacings and higher order reflections were calculated in each case and the values presented in Table II. As a matter of interest, Samples 28 and 29 were also heated to 550° for 30 min. and subsequently X-rayed. This latter procedure was undertaken to illustrate the reported collapse of montmorillonite under such conditions (10).

Table II.—X-Ray Diffraction Values of Selected Bentonites[a]

Sample No.					
1A	Untreated	11.26	4.03	3.13	..
	Glycolated	16.52	8.39	5.59	3.38
3	Untreated	11.63	4.02	3.11	..
	Glycolated	16.52	8.39	5.59	3.38
4	Untreated	10.72	4.76	3.15	..
	Glycolated	15.64	8.27	6.32	3.33
6	Untreated	11.05	3.15	..	..
	Glycolated	16.52	8.43	5.57	3.36
9	Untreated	11.19	3.13	..	..
	Glycolated	16.37	8.43	5.57	3.36
21	Untreated	11.79	4.93	3.11	..
	Glycolated	16.37	11.48	3.13	..
25	Untreated	12.11	3.11	..	..
	Glycolated	16.37	8.59	3.29	..
28	Untreated	10.59	4.80	4.01	3.14
	Glycolated	16.67	8.55	5.57	3.36
	Heated	9.46	4.75	4.04	3.16
29	Untreated	9.51	7.38	4.72	3.15
	Glycolated	15.64	8.27	6.33	3.33
	Heated	9.36	4.73	3.16	2.45

[a] Lattice spacings in Å.

DISCUSSION

The thermograms prepared in this series have generally similar shapes, although variation in certain details is observed. The initial endothermic

peak caused by loss of interlayer water occurs in all cases between 140 and 160°. Where present, the notch on recovery is attributed to the presence of calcium in the lattice. High temperature exotherms are not recorded because of difficulties encountered with the apparatus when temperatures were raised much above 800°.

Each sample was X-rayed before and after treatment with ethylene glycol, with the glycolated materials giving basal spacings slightly less than the accepted value of 17 Å. (11, 12). It was shown by Mackenzie (12) that this spacing would be reduced to lower values when insufficient ethylene glycol was present to complete the complex formation. However, before discarding the data, in question, a detailed study of the tracings was undertaken by Dr. L. B. Halferdahl.[1] While several of the clays showed quartz and/or gypsum peaks he was able to classify each of the samples as bentonite containing montmorillonite as the chief clay mineral. This finding, together with the evidence gathered from the differential thermal studies, was considered as sufficient to confirm the identity of the clays under study.

Further laboratory data designed to assist in evaluation of these clays as possible pharmaceutical agents will be reported in part II of this investigation.

[1] Mineralogist, Research Council of Alberta, Edmonton, Alberta.

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Pharmaceutical Investigation of Selected Alberta Bentonites II

Limit Tests and Mechanical Analysis

By ARTHUR J. ANDERSON† and ELMER M. PLEIN

A collection of bentonite samples from known Alberta deposits was examined. Limit tests as described in the B.P. and U.S.P. were completed on each. Additional procedures were undertaken to assist in evaluation of physical properties of the clays. On the basis of this work, one sample was shown to be of pharmaceutical quality, with a second sample from the same commercially important deposit being excluded from this category on a minor technicality only.

TWENTY-NINE SAMPLES of bentonite taken from deposits throughout Alberta were examined in the course of this work. All clays were identified as bentonite by qualified mineralogists, with verification by differential thermal and X-ray techniques being made on a selected group of interesting samples (1).

A laboratory study of the collected materials was then made to determine the presence of reserves of pharmaceutical grade bentonite in the province. A rigorous chemical analysis was not undertaken since pharmaceutical requirements were based mainly upon the inherent physical properties of the clays. However, certain chemical procedures were completed to aid in characterization.

EXPERIMENTAL

All samples were dried at 90°, reduced to a fine powder (200 mesh) by passage through a laboratory hammer mill, and then mixed well to obtain a uni-

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form blend. The materials were then stored in suitable closed glass, metal, or polythene containers.

After preliminary testing of several bentonites, a supply of Volclay B.C. grade[1] was chosen as most suitable for use as a control clay in this study.

Arsenic.—Since the B.P. (2) bentonite monograph contains a section limiting the maximum allowable arsenic content to not more than 8 p.p.m., it was felt that such data should be included in this study. For this purpose the quantitative procedure as detailed in the U.S.P. (3) was employed.

Nine Alberta clays, representative of deposits found in widely scattered areas of the province, were selected for this determination. Of these, seven clays yielded negative results, one a bare trace of color, and one a concentration of 1 p.p.m. of arsenic.

On the basis of the results obtained from the above sampling it was concluded that further testing was not indicated.

Alkalinity.—The B.P. states that a 2% w/v suspension of bentonite in distilled water should record a pH of 9.00–10.50. The U.S.P. requires a slightly narrower range of 9.00–10.00 for a 1 in 50 aqueous suspension.

Procedure

Two grams of clay was added in small portions to about 75 ml. deionized distilled water in a 100-ml. graduated cylinder. After all of the clay had been added and had settled to the bottom of the cylinder, more deionized water was added to the 100-ml. mark. The contents were then transferred to a Pyrex beaker containing a Teflon-coated stirring bar, and the assembly was placed on a magnetic stirrer. The contents were stirred at a rapid, uniform speed, and readings were taken with a Beckman Zeromatic pH meter using a calomel-glass electrode pair. The data are presented in Table I.

TABLE I.—pH VALUES OF ALBERTA BENTONITE AQUEOUS SUSPENSIONS[a]

Sample[b]	Mean	Range	Sample	Mean	Range
1	9.20	0.40	16	9.60	0.00
1A	8.87	0.20	21	9.15	0.00
3	8.93	0.12	23	9.70	0.00
4	9.35	0.10	24	9.83	0.05
5	9.27	0.05	25	9.70	0.00
6	9.75	0.02	26	9.49	0.02
7	8.54[c]	0.01	27	9.80	0.00
8	8.05[c]	0.10	28	9.00	0.00
9	8.80	0.00	29	9.50	0.00
10	9.03	0.20	30	9.22	0.05
11	8.60[c]	0.00	31	9.18	0.05
12	8.00[c]	0.15	32	9.37	0.05
15	8.62[c]	0.05			

[a] 2.0% w/v. [b] As indicated, certain samples were, for various reasons, discarded as unsuitable for laboratory testing. All clays were determined in triplicate with the exception of samples 8 and 23; in these two instances, duplicate readings only were recorded. [c] Considered as indicative of substandard bed.

Swelling Power.—To be acceptable as a pharmaceutical agent, a bentonite must show pronounced swelling ability upon hydration. The U.S.P. monograph describes a simple test designed to evaluate the swelling power of bentonite in distilled water. Readings are taken after 2 hours. The B.P. monograph sets a similar standard of quality. However, in this test the distilled water is replaced by a 1% sodium lauryl sulfate solution. The presence of the wetting agent assists materially in reducing the time needed to wet the powder. Furthermore, while the clay in this instance must also swell to the same apparent volume as before (24 ml.), the time required to do so is not specified.

The B.P. procedure was employed throughout in this determination. Clays were hydrated overnight and apparent volumes were read the next morning. Precautions were taken in each case to prevent loss of liquid through evaporation.

Six native bentonites were found to meet the official requirements of the test. However, a comparison of these values with those presented in Table I revealed little correlation between degree of alkalinity and swelling power. It was felt that this discrepancy could be due, in part at least, to excessive quantities of inert material in the native clays. Accordingly, a method for the removal of such substances was devised. This consisted of preparing 1 L. of a 4% (w/v) suspension of each clay sample in distilled water.[2] After sedimentation for 24 hours, the supernatant colloidal dispersion was removed by decantation and evaporated to dryness in an oven. The residue was then reduced to a 200-mesh powder and the B.P. test was employed, as before, to determine swelling power for each of the purified clays. Each sample was run in triplicate and the values averaged.

Apparent swelling volumes for each clay, before and after purification by water sedimentation, were compiled. These are presented in Fig. 1. Sample 7 could not be purified by this method since the total sample settled as "dead clay."

Mechanical Analysis.—During the preparation of bentonite dispersions it was observed that a marked difference in sand and silt content existed among the various samples. Since the presence of such inert material was considered undesirable in a pharmaceutical clay, a quantitative determination of sand, silt, and clay fractions of each sample was undertaken.

The U.S.D.A.[3] size limits for sand, silt, and clay fractions were employed (4). On this basis, 50 μ was taken as the dividing line between very fine sand and coarse silt. The lower limit for silt was set at 2 μ, and clay was considered to consist of all particles of diameters less then 2 μ.

Olmstead, Alexander, and Middleton (5) studied the application of the pipet method to mechanical analysis and developed an improved technique with a single pipet that was adapted by the Soils Laboratories of the U.S.D.A. Lyon and Buckman (6) suggested that the average surface soil could be considered to have a density of 2.65. Steele and Bradfield (7) later employed this factor to calculate sedimentation times at 25°. From this work it was determined that particles having a diameter of 2 μ and a density of 2.65 would require 41 minutes, 32 seconds at 25° to fall 1 cm. in a water medium.

[1] Received from the American Colloid Co., Merchandise Mart Plaza, Chicago 54, Ill.

[2] Because of their relatively high viscosity at this concentration, samples 1A, 21, 28, and 29 were further diluted to 2 L. to allow for satisfactory sedimentation.

[3] United States Department of Agriculture.

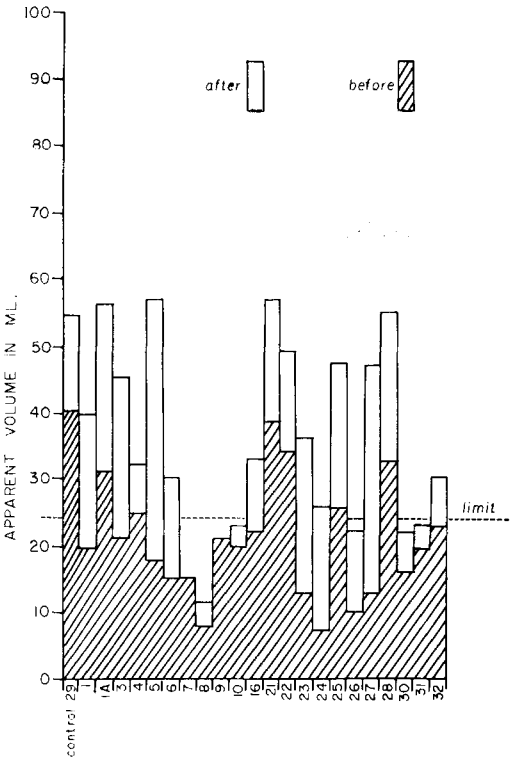


Fig. 1.—Swelling power of Alberta bentonites before and after purification by water sedimentation (2).

Procedure[4]

With the aid of a Waring Blendor, a 10-Gm. sample of air-dry clay was mixed to a smooth dispersion in 200 ml. of distilled water. The mixture was then transferred, with rinsings, to a 400-ml. electrolytic beaker, covered, and allowed to hydrate for 24 hours.

After hydration, 10 ml. of dispersing agent[5] was added to the system and the contents of the beaker were again transferred to a blending cup and stirred briskly for 20 minutes.

The dispersed sample was then washed onto a 300-mesh sieve,[6] the silt and clay passing through into a 1-L. graduated cylinder. Washing of the sieve contents was continued until the volume in the cylinder totaled 800 ml. Further washings were not collected, but the procedure was continued until the sand (and some coarse silt) was freed of any last traces of clay. Contents of the sieve were then washed into a tared aluminum dish, evaporated to dryness at 90°, and then dried to constant weight at 105°.

The silt and clay suspension was then made up to 1 L. with more distilled water, mixed well, and the cylinder placed in a suitable water bath at 25°. Sedimentation was calculated for 2-μ particles at 25° using the 2.65 density factor as recommended by Steele and Bradfield.

A 25-ml. automatic pipet with a filling time of 12 sec. was employed to obtain the clay aliquot at a depth of 8 cm. Filling of the pipet was accomplished by means of a controlled vacuum, and the contents were then transferred to a tared moisture dish. One rinse from the pipet was added and the dish was then placed in an oven at 90° until dry. Heating to constant weight was done at 105°.

All determinations were made at least in duplicate and reported in per cent on the basis of oven dry weight of the sample. In calculating the weight of clay obtained from the pipet aliquot, a correction factor was introduced to account for the weight of dispersing agent contained. The per cent of silt content was obtained by subtracting the sum of the percentages of sand and clay from 100. Values obtained are reported in Table II.

TABLE II.—PHYSICAL ANALYSIS OF ALBERTA BENTONITES

Sample[a]	Mean Sand Content, %	Range	Mean Clay Content, %	Range	Silt Content, % (By Difference)
1	1.52	0.03	82.17	0.07	16.31
1A	1.67	0.09	92.56	1.13	5.77
3	1.96	0.08	89.08	0.21	8.96
4	0.72	0.03	84.80	0.94	14.48
5	11.23	1.30	51.42	1.98	37.35
6	Trace	..	67.99	0.86	32.01
7	3.79	0.26	75.39	0.58	20.82
8	3.63	0.15	50.08	2.68	46.29
9	0.72	0.02	83.78	0.16	15.50
10	1.37	0.07	87.33	1.80	11.30
21	0.75	0.02	94.73	2.04	4.52
23	3.65	0.02	65.75	1.15	30.60
24	9.46	0.23	40.64	1.95	49.90
25	0.88	0.03	71.19	0.29	27.93
26	10.48	0.16	48.46	0.45	41.06
27	45.10	0.10	43.85	1.49	11.05
28	1.69	0.06	91.06	1.62	7.25
29	Nil	..	95.56	1.22	4.44
30	2.38	0.18	78.55	0.04	19.07
31	2.30	0.05	80.55	1.28	17.15
32	Nil	..	88.48	1.23	11.52

[a] Samples 29 and 32 were received as "grit free" powders due to their method of preparation.

Gel Formation.—Suspensions of swelling-type bentonite in suitable concentration will set to a gel, sometimes in a matter of seconds. Magnesium oxide has been shown to augment this effect significantly. An explanation of the phenomenon was later advanced by Hauser and Hirshon (8).

Both the B.P. and U.S.P. bentonite monographs contain provisions designed to set a minimum gel-forming standard for such clays. The test procedures are identical except for minor differences in wording. No more than 2 ml. of clear supernatant liquid should separate from the gel proper by the end of the test period.

For convenience, the B.P. procedure was followed in this study. Samples were prepared in triplicate, the results being averaged and presented in graphic form in Fig. 2.

[4] Based on the procedure employed in the Soils Laboratory, Research Council of Alberta, Edmonton, Alberta.

[5] Sodium hexametaphosphate, 35.70 Gm., and 7.94 Gm. of sodium carbonate per L. The sodium carbonate was employed as an alkaline buffer to prevent hydrolysis of the metaphosphate back to the *ortho* form.

[6] While the U.S.D.A. has set 50 μ as the lower limit of size for sand, a 300-mesh screen is usually employed for this purpose. Calibration of the screen used showed an average opening of 47.87 μ, indicating that at least a portion of the coarsest silt could be expected to remain in the sand fraction.

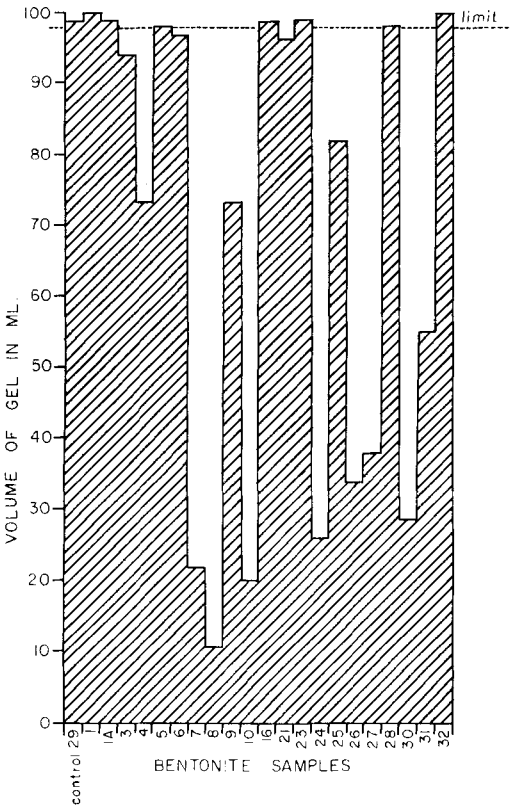


Fig. 2.—Gel formation of Alberta bentonites (2).

DISCUSSION

Alberta bentonites were found to be practically free of arsenic, making the clays suitable in this respect for use in medications to be taken internally. Alkalinity tests indicated that some samples were technically below standard. However, because the materials under test could not be considered as completely representative of each deposit, only five of the tabulated pH values were considered as probably indicative of substandard beds.

Swelling tests conducted on the clays indicate many are of the medium or low-swelling types. Six clays met official standards, four of which are from the Rosalind lease.[7] Preparation of clay concentrates by water sedimentation, with subsequent determination of swelling power of each, indicates that high-swelling fractions exist in a number of the known deposits. On that basis it would seem reasonable to anticipate that reserves of such high quality clay remain to be discovered in the province.

Twenty-one clays were examined for gel-forming ability. Seven of these were found to equal or exceed the official minimum requirements. It is interesting to note that of these, three clays had previously shown unsatisfactory swelling powers. Four of the seven samples which passed the gel-forming test were rich in clay content, but two of the seven were relatively poor in this respect. Such data only serve to accentuate the unpredictable nature of the native material.

On the basis of the official limit tests employed, only sample 28 was considered of pharmaceutical quality. Sample 1A, from the same beds was deficient in only one test, recording a pH of 8.87 instead of the required minimum of 9.00. Considering the method of collection, this deficiency could be considered a technicality.

While the B.P. "grit test" was not conducted on these materials, it is felt that the mechanical analysis procedure used was sufficiently stringent to afford an excellent indication of sand content to be found in the clays. Proper processing as a pharmaceutical grade bentonite would undoubtedly reduce this ingredient to a negligible quantity.

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[7] The Magcobar Mining Co. lease located at Rosalind, Alberta.

Rate Studies on the Hydrolysis of Niacinamide

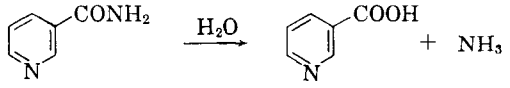
By PER FINHOLT† and TAKERU HIGUCHI

The apparent first-order rate constant corresponding to the hydrolysis of niacinamide has been determined over the pH range 0.4–11.3 in purely aqueous solutions at 89.4°. Although the pH-rate profile shows a minimum between pH 4 and 6, the reaction velocity is relatively slow over the usual pharmaceutical range. Surprisingly, the second-order proton catalyzed rate constants for the free vitamin and its protonated form appear to be roughly the same. The hydrolytic reaction appears to be subject to both general acid and general base catalysis.

NIACINAMIDE is one of the most stable of the vitamins in common use. It has been reported (1) that a 10% niacinamide solution in water may be autoclaved at 120° for 20 minutes without any degradation occurring. The great stability of niacinamide is probably the reason why there has been so little work done on the kinetics of the niacinamide hydrolysis. The only kinetic study reported in the literature was made by Jellinek and Gordon (2), who studied the hydrolysis in hydrochloric acid solutions. They found that it differed from the hydrolysis of other amides such as benzamide in that the first-order rate constants for the hydrolysis did not pass through a maximum between 3 and 4 *N* hydrochloric acid. There was a steady increase in the rate constant with increasing hydrochloric acid concentration from 0.05 *N* up to 8.4 *N*. Jellinek and Gordon did not study the hydrolysis in less acidic solution nor in alkaline medium.

The scope of the present work was to study the hydrolysis of niacinamide both in acidic and in alkaline solution, and to determine to what extent the hydrolysis is catalyzed by hydrogen and hydroxyl ions and by general acids and bases. It is known from the literature that the hydrolysis of amides may be subject to general acid base catalysis. As an example may be mentioned the hydrolysis of chloramphenicol, which is catalyzed by ammonia and ammonium ions, mono- and dihydrogen phosphate ions (3), undissociated acetic acid, mono- and dihydrogen citrate ions, and monohydrogen oxalate, succinate, tartrate, fumarate, phthalate, malonate, glutarate, and adipate ions (4–6).

It is assumed that the breakdown of niacinamide can be attributed solely to the hydrolytic cleavage of the amide linkage according to the equation



Received April 28, 1961, from the School of Pharmacy, University of Wisconsin, Madison.

Accepted for publication August 4, 1961.

This study was supported in part by a grant from the National Institutes of Health, Bethesda, Md.

† Sabbatical leave from the University of Oslo, Oslo, Norway.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

EXPERIMENTAL

Materials.—The niacinamide used was niacinamide U.S.P. from Mallinckrodt, recrystallized from benzene. All reagents used were of analytical grade.

Assay.—The study of the hydrolysis of niacinamide required an assay method which permitted an accurate determination of niacinamide in the presence of nicotinic acid. For the separation of the two compounds an ion exchange procedure recommended by Sweeney and Hall (7) was used According to this, nicotinic acid at pH 4.5–5 was quantitatively adsorbed by the anion exchange resin IRA-400, while niacinamide was not adsorbed and was determined in the eluate.

For the determination of niacinamide in the eluate, ultraviolet spectrophotometry was used. The absorption spectrum of niacinamide has a maximum at 262 mμ. The molar absorptivity is dependent on pH, as seen from Fig. 1. The protonated form of the amide has a higher molar absorptivity than the nonprotonated form. In order to convert the amide completely to its protonated form, 10% concentrated hydrochloric acid was added to the eluate before the reading in the spectrophotometer, a Cary recording model 11MS,

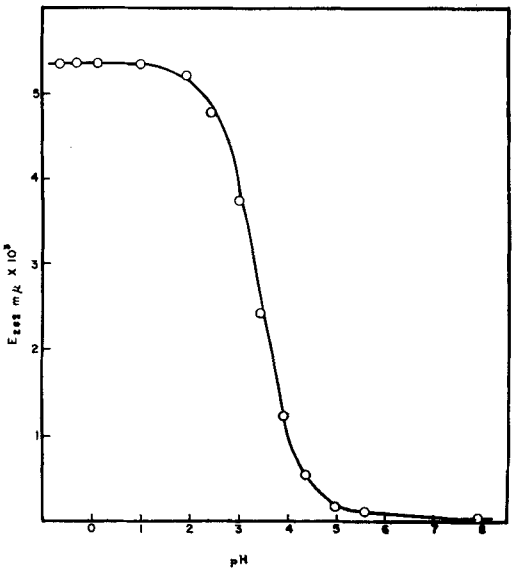


Fig. 1.—Plot showing the pH dependency of the molecular extinction coefficient of niacinamide at the absorption maximum at 262 mμ.

was made. A linear relationship between $A_{262m\mu}$ and concentrations of niacinamide ranging from 1 to 5 mg. in 100 ml. was found.

The complete assay method was as follows: An aliquot part of the sample, usually 2.00 ml., is added to the ion exchange column (Fig. 2) and adjusted to a pH of about 5. The column is washed with water. To the washings are added 10 ml. of the concentrated hydrochloric acid and water to 100 ml., and the absorbance measured in U.V. The content of niacinamide in the solution is read from a standard curve.

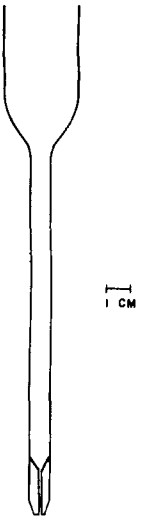


Fig. 2.—Ion exchange tube used for the separation of niacinamide and nicotinic acid.

By analyzing 30 samples with known content of niacinamide and niacin, 99–100.3% (average 99.6%) of the niacinamide was recovered. Sweeney and Hall found that about 0.8% of the niacinamide was hydrolyzed during the ionic exchange procedure. According to our results the amount hydrolyzed was even less. In no case will the hydrolysis on the column have any influence on the results of our kinetic studies.

High amounts of electrolytes in the solution on the column may, to some extent, displace the nicotinate ion from the column. With normal sodium chloride, for example, it was possible to elute nicotinic acid quantitatively. To analyze samples containing relatively high amounts of electrolytes it was, therefore, first necessary to determine the amount of the sample which may be collected through the column without getting any nicotinic acid in the eluate.

The procedure described proved to be satisfactory even in the presence of catechol, since this compound was firmly adsorbed by the resin.

Hydrolysis Experiments.—In most cases the following procedure was used: 122 mg. (0.001 *M*) of niacinamide was dissolved in 100 ml. of the appropriate buffer solution. The solution was placed in 5-ml. ampuls and heated to 89.4° in a constant temperature bath. Ampuls were taken out of the bath at appropriate intervals, cooled in ice, and the solution was analyzed. When a mixture of catechol and catecholate was used as buffer, the air in the ampuls was replaced by nitrogen.

In cases where the half-life was shorter than 2–3 hours, e.g., in strongly alkaline solution, the following technique was used: the alkaline buffer solution was heated to 89.4° in a 100-ml. volumetric flask in a constant temperature bath, and 122 mg. of niacinamide dissolved in 1 ml. of the same solution were added. Five-milliliter samples were withdrawn at appropriate intervals, mixed with a calculated amount of 50% sulfuric acid, and cooled in ice.

As far as those experiments are concerned where the main purpose was determination of the catalytic effect of a certain buffer, a series of solutions of different buffer concentrations but the same pH and ionic strength were required. To be able to calculate the composition of these solutions the pKa of the acid component in the buffer mixture was determined at 90° and at the actual ionic strength. The following equations were used for the actual calculations

At pH values less than 4

$$pH = pKa + \log \frac{[base] + [H^+]}{[acid] - [H^+]}$$

At pH values 4–8

$$pH = pKa + \log \frac{[base]}{[acid]}$$

At pH values greater than 8

$$pH = pKa + \log \frac{[base] - [OH^-]}{[acid] + [OH^-]}$$

[acid] and [base] are the measured amounts of the acid and the base component of the buffer mixture.

pH was determined at 90° with a Beckman expanded scale pH meter.

Determination of pKa of the Prontonated Form of Niacinamide.—The pKa of the protonated form of niacinamide was determined spectrophotometrically at room temperature, using the equation

$$pKa = pH - \log \frac{a_{NH^+} - a}{a - a_N}$$

where a_{NH^+} = molecular extinction coefficient of the protonated form of niacinamide, a_N = molecular extinction coefficient of the nonprotonated form of niacinamide, a = the observed molecular extinction coefficient, and a_{NH^+} and a_N were determined in solutions of pure NH^+ and N. A pKa of 3.40 was found.

pKa was determined electrometrically at 25° and at 90° ($\mu = 0.5$). The following values were found: pKa at 25° = 3.42 and pKa at 90° = 3.12.

RESULTS AND DISCUSSION

Order of Reaction with Respect to Niacinamide.—The rate of disappearance of niacinamide from the solutions was found to be strictly first order with respect to niacinamide at pH values from 0.4 up to 11.3. There was a linear relationship between time and logarithm of residual niacinamide concentration, as shown in Fig. 3.

Primary Salt Effect.—In strongly acidic solution the first step in the hydrolytic process was presumed

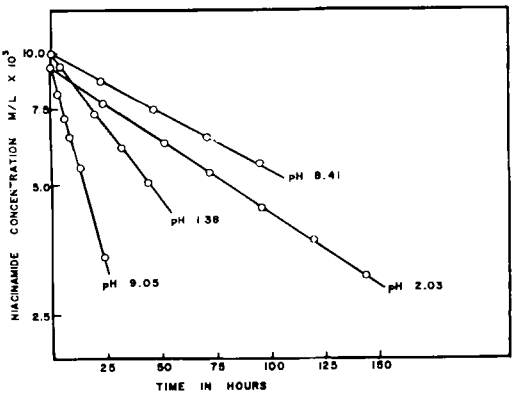
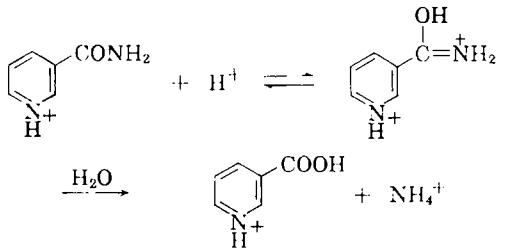


Fig. 3. Plots showing the overall first-order character of the hydrolysis of niacinamide at different pH values and 89.4°. For the actual runs 0.05*N* perchloric acid (pH 1.38), 0.5*M* phosphate (pH 2.03), 0.05*M* borate (pH 8.41), and 0.15*M* catecholate (pH 9.05) were used.

to be a reaction between hydrogen ions and the protonated form of the amide



Since a reaction between two charged species was involved in the hydrolytic process, a primary salt effect was expected. The existence of such an effect is obvious from Fig. 4 where the log of the rate constant k has been plotted against the square root of the ionic strength μ. The k values were found by making runs using 0.1 *N* hydrochloric acid or 0.02 *N* perchloric acid or 0.2 *N* perchloric acid to which different amounts of sodium chloride had been added. According to the theory of Brönsted and Bjerrum, there is a linear relationship between log k and the square root of the ionic strength when two charged species react in dilute solution ($\sqrt{\mu} < 0.2$). At higher concentrations the situation is more complex. It is therefore rather surprising that we find a nearly linear relationship between log K and $\sqrt{\mu}$ at $\sqrt{\mu}$ values from 0.2 up to 1, as shown in Fig. 4. Because of the primary salt effect in acid solutions all experiments in the acid region were carried out at the same ionic strength, namely $\mu = 0.5$.

In alkaline solution (pH 7–12) niacinamide will exist in the noncharged form. Experiments carried out in strongly alkaline solution showed, as expected, that there was no primary salt effect under these conditions. The same half-lives were found with 0.1 *N* sodium hydroxide as with 0.1 *N* sodium hydroxide containing 0.5 *M* sodium chloride (Fig. 5); and the same values were obtained with 0.025 *N* barium hydroxide as with 0.025 *N* barium hydroxide containing 0.5 *M* sodium chloride.

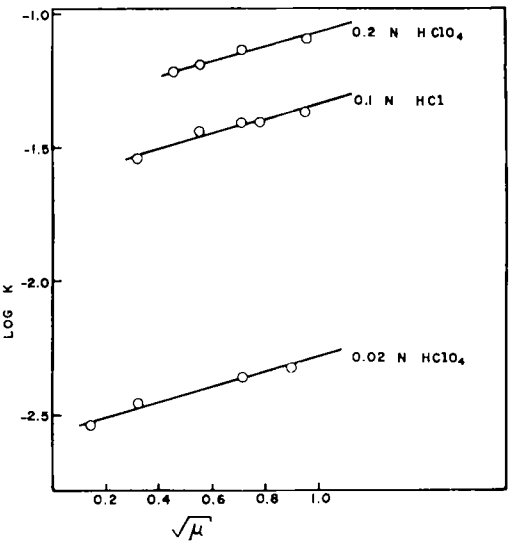


Fig. 4. Plots showing the effect of the ionic strength (μ) on the rate of niacinamide hydrolysis in strong acids at 89.4°. The ionic strength of the acids was varied by addition of different amounts of sodium chloride.

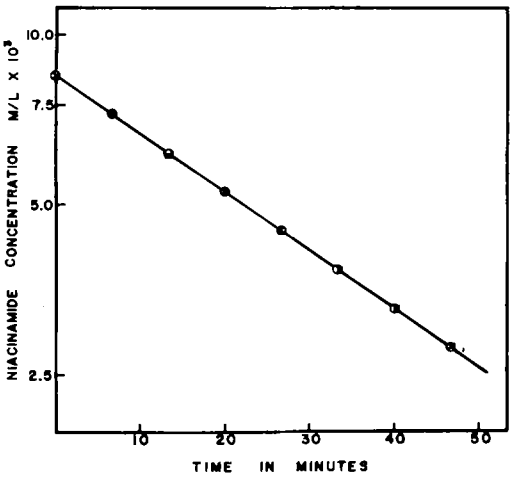


Fig. 5.—Plot showing the rate of niacinamide hydrolysis in 0.1*N* sodium hydroxide (o) and in 0.1*N* sodium hydroxide containing 0.5*M* sodium chloride (x). The coincidence of the experimentally determined points shows that the rate of niacinamide hydrolysis is independent of ionic strength.

Catalytic Effect of Hydrogen Ions and Hydroxyl Ions on the Hydrolysis of Niacinamide. The catalytic effect of hydrogen ions on the hydrolysis of the protonated form of niacinamide was determined by using hydrochloric acid or perchloric acid ($[H^+] \gtrsim 0.1$) as solvents. See Table I. At these high hydrogen ion concentrations niacinamide will exist only in the protonated form.

The catalytic effect of hydroxyl ions was determined by using solutions of sodium hydroxide or barium hydroxide as solvents. See Table II.

Catalytic Effect of General Acids and Bases on the Hydrolysis of Niacinamide. The catalytic

TABLE I.—CATALYTIC EFFECT OF HYDROGEN IONS ON THE HYDROLYSIS OF THE PROTONATED FORM OF NIACINAMIDE

Acid	[H+]	k, hr.$^{-1}$ × 10^2	k_{H+},[a] hr.$^{-1}$ mol.$^{-1}$ L. × 10
HCl	0.10	3.41	3.41
HCl	0.50	19.4	3.88
$HClO_4$	0.20	6.79	3.40

[a] Average: k_{H+} = 3.6 × 10^{-1} hr.$^{-1}$ mol.$^{-1}$ L.

TABLE II.—CATALYTIC EFFECT OF HYDROXYL IONS ON THE HYDROLYSIS OF NIACINAMIDE

Base	[OH−]	k, hr.$^{-1}$ × 10	k_{OH-},[a] hr.$^{-1}$ mol.$^{-1}$ L.
NaOH	0.05	6.87	13.7
NaOH	0.10	14.0	14
NaOH	0.20	32.8	16.4
$Ba(OH)_2$	0.02	2.75	13.8
$Ba(OH)_2$	0.025	3.16	12.6
$Ba(OH)_2$	0.05	6.87	13.7
$Ba(OH)_2$	0.10	17.7	17.7

[a] Average: k_{OH-} = 14.6 hr.$^{-1}$ mol.$^{-1}$ L.

effect of certain buffers was determined by making runs with each buffer, varying the total buffer concentration but keeping the pH and the ionic strength constant in each series. By plotting the observed k values against buffer concentration, straight lines were obtained, the slopes of which were a measure of the catalytic activity of the buffers.

Figure 6 shows that phosphate buffers with pH 10.19 and pH 10.33 have a catalytic effect on the hydrolysis of niacinamide. At pH 8.64 the effect is very small. The phosphate buffers with pH 8.64 were solutions of Na_2HPO_4. The catalytic constant of HPO_4^- is obviously very small compared to the catalytic constant of $PO_4^{\equiv}$. The small slope of the line corresponding to pH 8.64 may be attributed partly to the amount of $PO_4^{\equiv}$ ions present in the Na_2HPO_4 solutions.

For the hydrolysis of niacinamide in the actual phosphate buffers the following equation is valid

$$k = k_0 + k_{HPO_4^-}[HPO_4^-] + k_{PO_4^{\equiv}}[PO_4^{\equiv}] \qquad \text{(Eq. 1)}$$

At pH 10.33 and pH 10.19 $k_{HPO_4^-}[HPO_4^-]$ is negligible compared to $k_{PO_4^{\equiv}}[PO_4^{\equiv}]$ and may be omitted, thus giving

$$k = k_0 + k_{PO_4^{\equiv}}[PO_4^{\equiv}] \qquad \text{(Eq. 2)}$$

k_0 is equal to k at zero buffer concentration, i.e., equal to the intercept of the lines with the y-axis.

$[PO_4^{\equiv}]$ may be calculated from the equation

$$\frac{[PO_4^{\equiv}]}{C - [PO_4^{\equiv}]} = \frac{ka_3}{[H^+]} \qquad \text{(Eq. 3)}$$

where C = total buffer concentration and ka_3 = $10^{-10.72}$ (89.4° and μ = 0.75).[1]

Using Eqs. 2 and 3, the run at pH 10.33 gives $k_{PO_4^{\equiv}}$ = 6.8, the run at pH 10.19 gives $k_{PO_4^{\equiv}}$ = 6.5. Average $k_{PO_4^{\equiv}}$ = 6.65 hr.$^{-1}$ mol.$^{-1}$ L.

Figure 7 shows the catalytic effect of phosphate buffers pH 6.40–7.70. There is an increase in k with increasing buffer concentration especially at

[1] A 0.2 M phosphate buffer pH 10.19 has μ = 0.74.

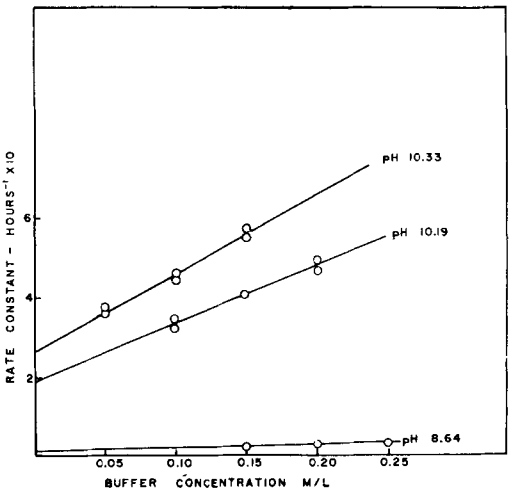


Fig. 6.—Plots showing the effect of phosphate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.75.

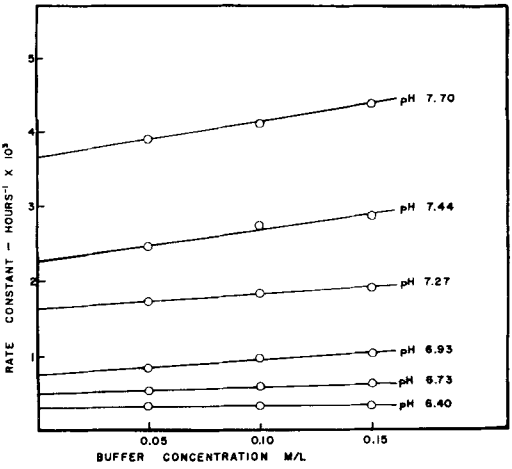


Fig. 7.—Plots showing the effect of phosphate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.75.

the highest pH values. The catalytic effect of these buffers seems, however, to be mainly due to the catalytic effect of the $PO_4^{\equiv}$ ions present. The following calculation indicates this.

At pH 7.7 the increase in k going from zero buffer concentration up to 0.15 M is 0.72 × 10^{-3}. At pH 7.7 $[PO_4^{\equiv}]$ = 1.26 × 10^{-4} calculated according to the equations

$$[PO_4^{\equiv}] = \frac{ka_2 \times ka_3}{[H^+]^2}[H_2PO_4^-]$$

$$\frac{0.15 - [H_2PO_4^-]}{[H_2PO_4^-]} = \frac{ka_2}{[H^+]}$$

where ka_2 = $10^{-6.45}$ (90° and μ = 0.75). $k_{PO_4^{\equiv}}$ × $[PO_4^{\equiv}]$ = 6.65 × 1.26 × 10^{-4} = 0.84 × 10^{-3}, i.e., nearly equal to the increase in k.

A similar calculation using the results from the experiments at the lower pH values show that in most cases the increase in k is a little higher than what would have been expected if $PO_4^{\equiv}$ was the only catalytic species.

The catalytic effect of $H_2PO_4^-$ is shown in Fig. 8. If we assume that the increase in k may be attributed solely to a catalytic effect of $H_2PO_4^-$ on the nonprotonated form of niacinamide, calculation gives as result

$$k_{H_2PO_4^-} = 4.4 \times 10^{-4} \text{ hr.}^{-1} \text{ mol.}^{-1} \text{ L.}$$

Figure 9 seems to indicate that $H_2PO_4^-$ has a more pronounced catalytic effect on the hydrolysis of the protonated form of niacinamide than on the hydrolysis of the nonprotonated form. There also seems to be a catalytic effect of the undissociated phosphoric acid on the protonated form of the amide.

Higuchi and collaborators (6) have shown that the "half salt" of dibasic carboxylic acids, such as oxalic, succinic, tartaric, fumaric, phthalic, malonic, glutaric, and adipic acid has a strong catalytic

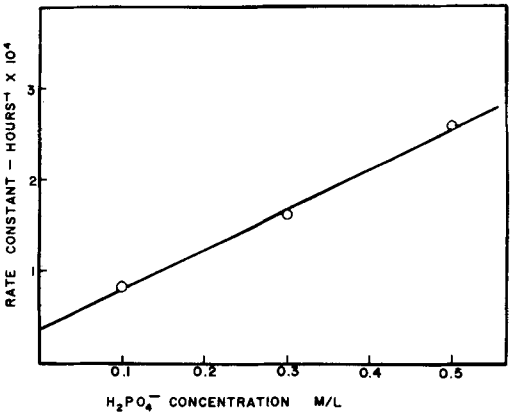


Fig. 8.—Plot showing the effect of dihydrogen phosphate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at 89.4° and at ionic strength of 0.5 and pH-4.50.

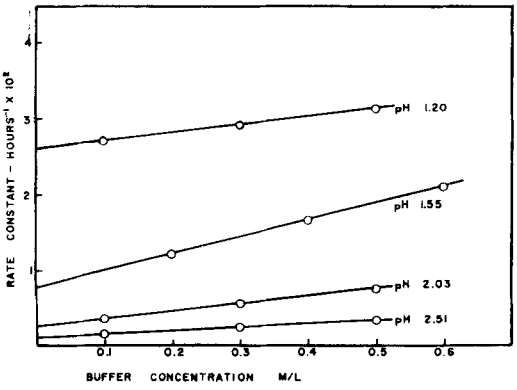


Fig. 9.—Plots showing the effect of phosphate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.5.

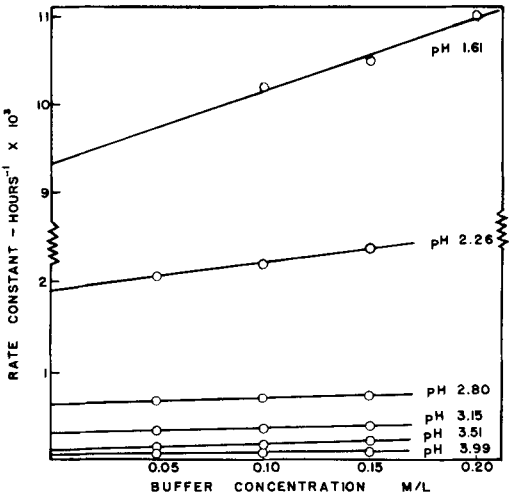


Fig. 10.—Plots showing the effect of oxalate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.5.

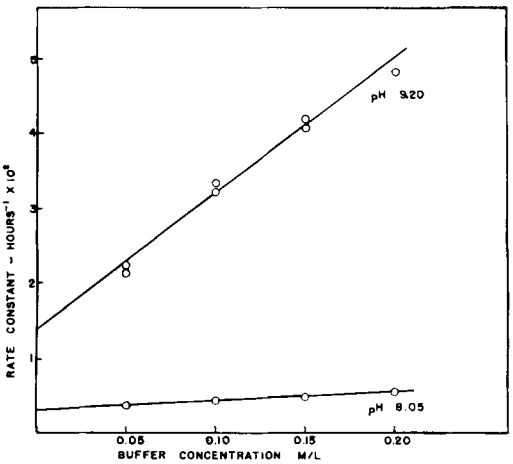


Fig. 11.—Plots showing the effect of catecholate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.2.

effect on the hydrolysis of chloramphenicol. A similar effect could not be demonstrated for the "half salt" of oxalic acid on the hydrolysis of niacinamide (Fig. 10, curve marked pH 2.80). With decreasing pH, i.e., with an increase in the concentration of undissociated oxalic acid and of protonated niacinamide, there is a slight increase in the catalytic effect of the oxalate buffers.

Catechol catalyzes, according to Higuchi and collaborators (6), the hydrolysis of phosphonate esters. In the present work the influence of catechol on niacinamide hydrolysis has been studied (Fig. 11). The great difference in slope between the two lines on Fig. 11 seems to indicate that the catalytic effect of the catecholate buffers must be attributed mainly to the double charged catechol

anion. pKa of catechol was determined to be 8.65 at 89.4° and $\mu = 0.2$, which was the ionic strength used in the hydrolysis experiments.

The evaluation of the catalytic effect of borate buffers gives rise to certain problems, because these buffers do not contain only undissociated H_3BO_3 and $H_2BO_3^-$ ions, but also the ions BO_2^- and $B_4O_7^=$. The actual concentration of each of these ions cannot be determined and it is therefore impossible to make an accurate calculation of the ionic strength of the buffer solutions and to calculate the catalytic constant of each species. The first of the difficulties mentioned may be handled by using the buffer in a relatively low concentration and adding a relatively great amount of a neutral salt. The ionic strength will then be determined mainly by the neutral salt. In our experiments the total buffer concentration never exceeded 0.15 *M* and sodium chloride was added to $\mu = 0.75$.

pKa of boric acid was determined to be 8.42 at 90° and $\mu = 0.75$. It is obvious from Fig. 12 that the catalytic effect of borate buffers is due mainly to the "borate" ions, not to undissociated boric acid. A calculation of the catalytic constant of the borate ions, assuming they all exist as $H_2BO_3^-$, gave results varying from 0.7×10^{-1} (at pH 7.65) up to 1.2×10^{-1} (at pH 8.77).

pH-Rate Profile of Niacinamide Hydrolysis.—The pH-rate profile (Fig. 13) shows the relation between pH and log k at zero buffer concentration. pH of the strongly acid solutions at 89.4° and $\mu = 0.5$ were calculated from the equation

$$\text{pH} = -\log [H^+] + 0.08$$

The above relationship between pH and $[H^+]$ was obtained as the result of pH measurements in acids of known hydrogen ion concentration at 89.4° and $\mu = 0.5$. In a similar manner we found the following relationship between pH and $[OH^-]$ at 89.4° and $\mu = 0.75$

$$\text{pH} = 12.0 + \log [OH^-]$$

This equation was used for calculating the pH of the strongly basic solutions.

In the alkaline region the phosphate and borate buffers were adjusted to an ionic strength of 0.75. The ionic strength of the catecholate buffers was 0.20. The pH of these buffers at $\mu = 0.75$ were found to be equal to the pH at $\mu = 0.20$ less 0.15.

At zero buffer concentration the following reactions will contribute to the overall velocity of the hydrolysis

$N^+ + H^+ \rightarrow$ products, rate constant: k_{H^+}'
$N + H^+ \rightarrow$ products, rate constant: k_{H^+}''
$N^+ + H_2O \rightarrow$ products, rate constant: k_{H_2O}'
$N + H_2O \rightarrow$ products, rate constant: k_{H_2O}''
$N^+ + OH^- \rightarrow$ products, rate constant: k_{OH^-}'
$N + OH^- \rightarrow$ products, rate constant: k_{OH^-}''
N^+ = protonated form of niacinamide
N = nonprotonated form of niacinamide
$[N_T] = [N] + [N^+]$

The overall velocity is equal to the sum of the rates of all these reactions

$$-\frac{d[N_T]}{dt} = k_{H^+}'[N^+][H^+] + k_{H^+}''[N][H^+] + k_{H_2O}'[N^+][H_2O] + [k_{H_2O}''][N][H_2O] + [k_{OH^-}'][N^+][OH^-] + [k_{OH^-}''][N][OH^-] \quad \text{(Eq. 4)}$$

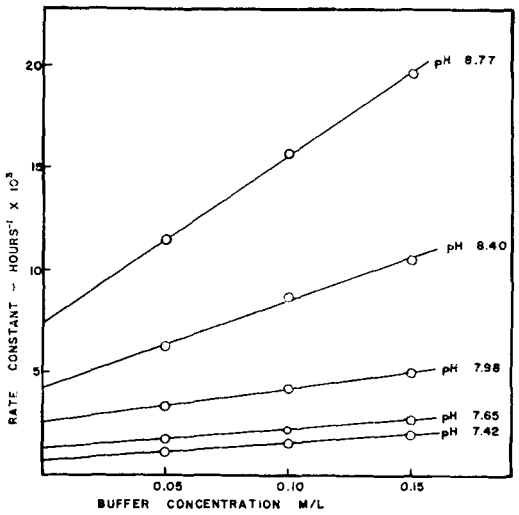


Fig. 12.—Plots showing the effect of borate concentration on the pseudo first-order rate constant of niacinamide hydrolysis at fixed pH values. All the runs were made at 89.4° and at ionic strength of 0.75.

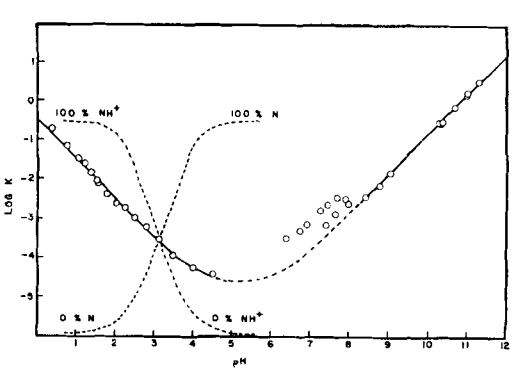


Fig. 13.—Plot showing the relation between pH and the log of the pseudo first-order rate constant of niacinamide hydrolysis at 89.4° and zero buffer concentration. The dotted S-shaped curves show the amount of the protonated (NH^+) and nonprotonated (N) form of niacinamide in relation to pH.

In fairly strongly acidic solution the last four terms of Eq. 4 will be negligible compared to the first two terms and may be omitted, thus giving

$$-\frac{d[N_T]}{dt} = k_{H^+}'[N^+][H^+] + k_{H^+}''[N][H^+]$$

Because of the overall first-order character of the hydrolysis the following equation is valid

$$-\frac{d[N_T]}{dt} = K[N_T] \quad \text{(Eq. 5)}$$

Combining the last two equations and the equation

$$k_a = \frac{[N][H^+]}{[N^+]}$$

gives

$$k = [H^+]\frac{k_{H^+}'[H^+] + k_{H^+}''(K_a)}{[H^+] + k_a}$$

The fact that the pH-rate profile is a nearly straight line from pH 0.4 to pH 3.5 shows that k_{H^+}' and k_{H^+}'' must be of the same order of magnitude, thus giving

$$k = [H^+][k_{H^+}'] = [H^+]\, k_{H^+}''$$

$$\log k = -pH + 0.08 + \log k_{H^+}'$$

If k_{H^+}'' had been different from k_{H^+}' there would have been a break in the curve around pH = pKa = 3.1.

In fairly strongly basic solution the five first terms in Eq. 4 may be omitted. Doing this and combining with Eq. 5 gives

$$k = k_{OH^-}''\,[OH^-]$$

$$\log k = pH - 12 + \log k_{OH^-}''$$

In accordance with this last equation the pH-rate profile is a straight line between pH 8.4 and 11.3. Between pH 6.4 and 8 however, the experimentally determined k values deviate from the straight line. The deviations were greatest when phosphate was used as buffer [the 6 points lying farthest away from the dotted line (Fig. 13) correspond to k values obtained from experiments with phosphate as buffer]. The deviations were relatively small when borate was used as buffer (corresponding to the 3 points closest to the dotted line). With catechol catecholate as the buffer, the deviation was intermediate (the point with the coordinates 7.9–2.5).

In the pH region 6.4–8 the data suggest that reactions other than the hydrolysis catalyzed by hydroxyl ions and the buffers may participate significantly. It is difficult to say what these reactions are. One must keep in mind, however, that in the pH region 6.4–8 the half-life of niacinamide is very long, 10–100 days. This means that the niacinamide solutions in our experiments had to be heated at 89.4° from one to several weeks. During this long period of heating the solution will attack the glass surface and extract products such as silicates and metals, which may well have a catalytic effect. Phosphates are known to be especially aggressive against glass. In addition to this, the glass surface itself may act as a catalyst, thus promoting a heterogeneous catalysis.

The pH-rate profile seems to level off earlier than would have been the case if k_{H_2O}' and k_{H_2O}'' had been negligible. This means that at pH 4.5–6 the overall velocity is mainly determined by the noncatalyzed hydrolysis.

SUMMARY AND CONCLUSIONS

1. The hydrolysis of niacinamide was found to be first order with respect to niacinamide over a wide range of hydrogen ion concentration (pH 0.4–11.3).

2. The rate of the hydrolysis has a minimum at pH 4.5–6. In this region, the half-life of niacinamide was about 1000 days at 89.4°.

3. The hydrolysis is catalyzed by hydroxyl ions and hydrogen ions. The catalytic constant of the hydroxyl ions was found to be $k_{OH^-} = 14.6$ L. hr.$^{-1}$ mol.$^{-1}$ at 89.4°. The catalytic effect of the hydrogen ions was determined to be nearly the same for the hydrolysis of the protonated form of niacinamide as for the hydrolysis of the nonprotonated form, $k_{H^+} = 3.6 \times 10^{-1}$ L.hr.$^{-1}$ mol.$^{-1}$ at 89.4°.

4. The hydrolysis is catalyzed by general acids and bases. The highest effect was produced by the triply charged phosphate ion. Considerable effects by the doubly charged catechol ion and the borate anions were also noted. Mono- and dihydrogen phosphate ions were found to have a very small effect on the hydrolysis of the nonprotonated form of niacinamide. Monohydrogen phosphate seemed to have a somewhat stronger effect on the hydrolysis of the protonated form. The hydrolysis of this form also appeared to be slightly catalyzed by undissociated phosphoric acid and oxalic acid.

5. A positive, primary salt effect was noted in strongly acidic solution; but none in strongly alkaline solution was noted.

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Immunochemical Study of the Diphtheria Toxin-Fluorescent Antitoxin System

By W. F. HEAD, Jr.†

Six fluorescent diphtheria antitoxins were prepared. These products were then examined for excess adsorbed fluorescein, chemically bound fluorescein, and reactivity toward diphtheria toxin and toxoid. Particular attention was paid to antitoxic potency loss inasmuch as it was desired to find optimum methods of preparation wherein maximum labeling and, therefore, higher sensitivity could be obtained with maximum retention of activity. The fluorescence of the floccules formed at the equivalence point in the immunochemical titration of toxin with fluorescent antitoxin was shown to be directly proportional to the biological potency of the toxin.

COONS and Kaplan (1) were the first to demonstrate the fluorescent antibody technique by determining the localization of specific antigens in tissues using the homologous fluorescent antibody. Many similar diagnostic tests have appeared in the literature (2–7) since that time; however, only empirical data are available for preparation of fluorescent antibodies and quantitative information concerning the antigen-fluorescent antibody reactions is lacking entirely.

The excellent sensitivity afforded by fluorescence measurements combined with the high degree of specificity involved in antigen-antibody reactions would seem to justify examination of quantitative data for possible application as an analytical method. The principle of such a quantitative procedure rests upon the proportionality of the biological potencies of various toxins or toxoids to the weights of antigen-antibody complexes at the equivalence points in their titrations. It is also necessary that the ratio of antigen to antibody at these points be constant. If these requirements are met, a randomly fluoresceinated antibody should carry amounts of fluorescein into the precipitated complex which are proportional to potency. This has been accomplished in connection with antibody-dye complexes on a colorimetric basis (8).

EXPERIMENTAL

Materials.—Diphtheria antitoxin concentrate (Lilly lot No. 729077) was used for the preparation of all fluorescent antitoxins. This material had a potency of 3,800 guinea pig protective units per ml., 3,700 *Lf* (flocculating) units per ml., and a *Kf* value of 4 minutes. The total protein content, chiefly gamma globulin, was 20%. The antitoxin was of equine origin. Various diphtheria toxins and toxoids were also supplied by Eli Lilly and Co.

Fluorescein amine and fluorescein isothiocyanate were obtained from Nutritional Biochemicals Corp. Reagent grade formamide was purchased from Matheson, Coleman, and Bell.

Preparation of Fluorescent Antitoxins.—The initial preparation was made by coupling fluorescein isocyanate to the antibody molecule as previously described (1). The dialysis purification was carried out at room temperature until no appreciable fluorescence was observed in the external fluid (17 to 20 days). The product was adjusted to a convenient volume with 0.9% saline and made 1:10,000 in thimerosal. All subsequent preparations were made by essentially the same process with the exception of using fluorescein isothiocyanate and maintaining a cold temperature, 4 to 6°, throughout the process, including the dialysis.

Titration of Diphtheria Toxin or Toxoid with Homologous Fluorescent Antitoxin.—A classical Ramon titration was employed wherein small increments of antitoxin were added to constant amounts (0.5 ml.) of toxin or toxoid in several tubes. The tubes were brought to equal volume, 2 to 4 ml. depending on antitoxin potency, with phosphate buffer, pH 7.4, 0.066 *M*, then either incubated at 50° for 2 hours and stored overnight under refrigeration or incubated overnight at 50° and allowed to stand an additional day at room temperature to reach equilibrium. These procedures were used to insure quantitative precipitation of floc. Usually, toxin flocs were refrigerated and toxoid flocs were developed at room temperature. It was found that toxoid flocs with fluorescent antitoxin were partially redissolved when cold, and additional floc would form as the tubes warmed to room temperature. The precipitates were separated by centrifuging and were resuspended and washed once with 1 to 3 ml. of 0.9% saline to remove residual unreacted fluorescent material.

Measurement of Floc Fluorescence.—In order to measure the fluorescein, the flocs had to be redissolved after washing. A study was undertaken to find the appropriate solvent. Of 25 solvents tested, formamide proved to be the most acceptable, dissolving large quantities of floc and simultaneously enhancing fluorescence because of its basic qualities.

The flocs were dissolved with either 3 or 4 ml. of formamide. Fluorescent intensity measurements

Received June 14, 1961, from the Analytical Research Department, Eli Lilly and Co., Indianapolis, Ind.
Accepted for publication August 7, 1961.
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Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
Grateful thanks are given to Mr. Max M. Marsh for his interest, encouragement, and aid in this work. To Mr. Robert Jeffery and Dr. Roland Hodgson (deceased), a debt of gratitude is also due for supplying the various biologicals and performing some of the tests.

were taken with an Aminco-Bowman spectrophotofluorometer using an activation wavelength of 490 mμ. The emission intensity at 525 mμ was recorded with the appropriate sensitivity setting. To standardize the instrument, a formamide solution of fluorescein, containing 0.333 mcg./ml., was used. A typical fluorescent floc emission spectrum is shown in Fig. 1.

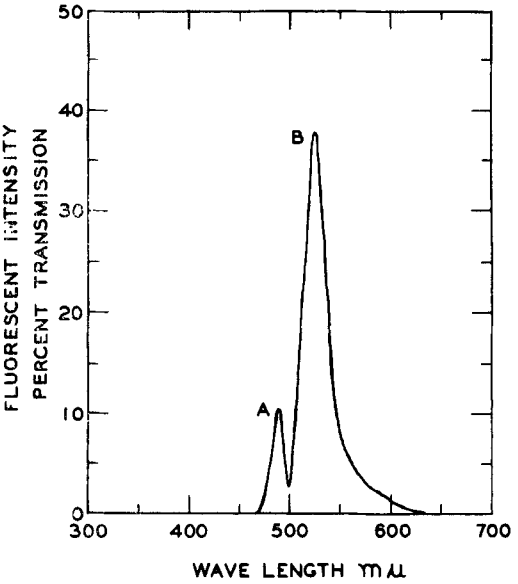


Fig. 1.—Emission spectrum of a fluorescent diphtheria floc. Activation 490 mμ. A, Activation crossover peak; B, fluorescent emission peak.

Standard Curves.—The fluorescent intensities of all flocs, adjusted to the same instrumental sensitivity setting, were plotted *vs.* the corresponding flocculating units of antitoxin added. These curves, as seen in Fig. 2, have the general shape of the diphtheria precipitin curve obtained by weight or nitrogen analysis. If toxins or toxoids of different potencies were used, a plot of the various maximum fluorescent intensities at the equivalence points in the individual titration curves *vs.* potency, either in *Lf* units or guinea pig protective units, would be linear with zero intercept. The slope would be determined by the degree of fluorescein labeling of the antitoxin. This concept is shown in Fig. 3. Construction of these standard curves with each fluorescent antitoxin was not necessary for quantitative potency determinations; it was even more accurate to test a standard toxin along with an unknown and compare equivalence point floc fluorescence and potency on a proportional basis.

Comparison Analytical Method.—The standard *Lf* test was used as a reference method with which to compare the fluorescent antibody method and to determine flocculating power of various antigens and antitoxins. This method is a titration similar to that already described using small increments of standard antitoxin or toxin. The tubes were incubated at 50° and constant observation maintained until the first floccules appeared. The tube showing the first trace of floc is the equivalence point tube and the time required for flocculation

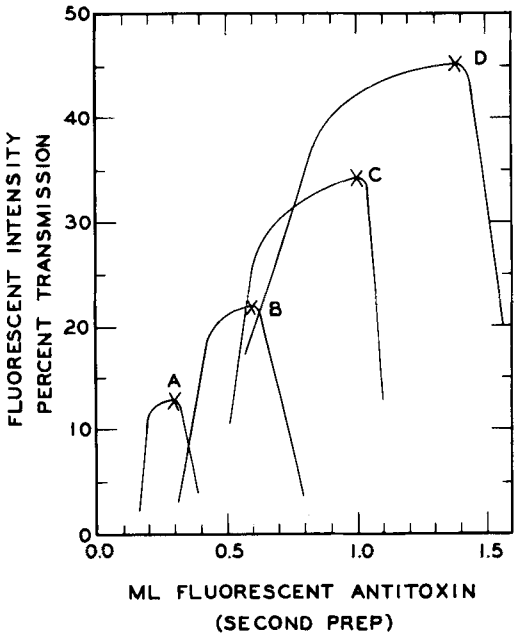


Fig. 2.—Precipitin curves obtained by measurement of floc fluorescence. Curve A, diphtheria toxin potency, 27.5 *Lf*/ml.; curve B, diphtheria toxin potency, 55 *Lf*/ml.; curve C, diphtheria toxin-potency, 82.5 *Lf*/ml.; curve D, diphtheria toxin potency, 110 *Lf*/ml.; × indicates the equivalence points.

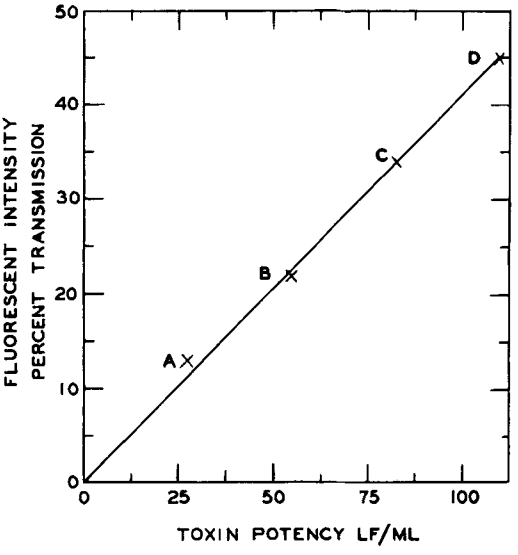


Fig. 3.—Equivalence point fluorescence (Fig. 2) *vs.* toxin potency. A, B, C, D, toxin potencies (same as Fig. 2).

to occur is the *Kf* value. This value is indicative of the degree of denaturation, a longer time being required for flocculation by a more highly denatured toxin or antitoxin. The standard antitoxins or toxins were National Institutes of Health primary standards or secondary standards derived from them.

TABLE I.—CHARACTERISTICS OF VARIOUS FLUORESCENT ANTITOXINS

Prepn.	Potency Loss (from Original) Toxin, %	Potency Loss (from Original) Toxoid, %	Reactants: Fluorescein Derivative, mg.	Reactants: D.A.T.C.,[a] ml.	*Kf*, min.	Inert Adsorbed Fluorescein	S.P.N.,[b] %	Fluorescein Ratio[c]
No. 1	58	100	50	5	25	[d]	[d]	[d]
No. 2	28.5	100	50	5	15	Yes	9.36[e]	5.35
No. 3	20.5	100	50	10	10	Yes	10.44[e]	5.4
No. 4	44	44	[f]	[f]	8	No	5.14	5.25
No. 5	0	0	4	10	5	Very slight	5.54[g]	1.5
No. 6	0	0	20	10	5	Yes	5.76	4.25

[a] Diphtheria antitoxin concentrate. [b] Specifically precipitable nitrogen. [c] Number of fluorescein molecules per antitoxin molecule. [d] Not determined. [e] These values determined with use of toxin, giving greater precipitability than the last three, which were determined with the use of toxoid. [f] Preparation made from No. 3. [g] Nitrogen content per *Lf* toxoid, used in calculating this value, was calculated from data obtained from the reaction of No. 4 and No. 6 antitoxins with the same toxoid.

Determination of Specifically Precipitible Nitrogen and Fluorescein to Antitoxin Ratios.—Since diphtheria antitoxin concentrate contains a considerable amount of inert globulin, gross fluorescence and total protein measurements will not give much information concerning that fluorescein bound to antitoxic globulin molecules. To obtain these data, a method described by Kabat and Mayer (9) was used. This technique enables the determination of the amount of antitoxin precipitated. Fluorescence measurements of the dissolved flocs reveal the amount of fluorescein present. From these figures, the degree of labeling is easily calculated. The molecular weight value of the antitoxin molecule is 184,000 (10).

RESULTS AND DISCUSSION

First Fluorescent Antitoxin.—By means of *Lf* tests, this material was found to have lost 58% of its potency against toxin and 100% of its antitoxoid activity. The *Kf* value was 25 minutes. In addition to potency loss, only a slight similarity to typical precipitin curves could be obtained. The material, after standing for a few weeks under refrigeration, deposited an appreciable amount of granular fluorescent material and was considered to be an altogether unsatisfactory preparation. A summary of the proportion of reactants and the characteristics of this and all other fluorescent antitoxins prepared is given in Table I.

Second Fluorescent Antitoxin.—Toxoid flocculating power was again destroyed, but 72.5% of the toxin flocculating ability was retained. The *Kf*, being 15 minutes, was still 11 minutes longer than the original unconjugated antitoxin. A standard curve was prepared using this antitoxin with good linearity and zero intercept. Twenty-five *Lf*, corresponding to approximately 0.25 mg. toxin protein, was the smallest amount of toxin that could be accurately assayed. Since the *Kf* of this fluorescent antitoxin was still comparatively long, it was possible that excess fluorescein had been used and was occupying specific reactive sites in the molecule without contributing materially to the fluorescence of the flocs.

Third Fluorescent Antitoxin.—The method of preparation was the same as the method employed for the second fluorescent antitoxin except that twice as much diphtheria antitoxin concentrate was used. Again, 100% toxoid activity was lost, but only 20.5% toxin activity was lost. The *Kf* decreased to 10 minutes. A standard curve was prepared using dilutions of toxin as before. Surprisingly, the slope of this curve was almost identical with the slope of the curve obtained with the second preparation instead of being approximately one-half as great. When the overall fluorescence of this third antitoxin was compared to the fluorescence of the second preparation, the latter showed appreciably more gross fluorescence by a ratio of 82 to 49. This greater fluorescence must be accounted for in some immunochemically inactive manner. Either this fluorescein is bound to the inert globulin present or is physically adsorbed on the globulin and antitoxin and is released upon reaction. The latter seems much more probable since the two proteins have no apparent chemical differences other than the spatial arrangement of polypeptide chains. Verification of this was obtained by setting up tests using identical quantities of toxin and titrating with the second and third antitoxin. The floc fluorescence and the fluorescence of the supernatants were measured. It was found that floc fluorescence was the same for both materials and the fluorescence of the supernatants was in the same approximate ratio as the unreacted antitoxins. Control tubes of toxin, antitoxin, and buffer were mixed in the same proportion as in the floc tests immediately preceding the fluorescence measurement. Aliquots were taken from these tubes before flocculation could occur and in this manner served to compensate for any buffer effects, slight toxin fluorescence, etc., that might be misinterpreted. The ratios of fluorescence for the two antitoxins were 1.65 for unreacted or unflocculated controls and 1.58 for the supernatants after flocculation.

By using the control tests, a greater amount of fluorescence was consistently observed in the supernatant fluids, even after removal of some fluorescein in the flocs, than was present in the unflocculated control tubes. This could be explained by the adsorption of fluorescein to the antitoxin surface and in some manner not exhibiting fluorescence. During the reaction with toxin the fluorescein could be liberated into solution This bound fluorescein might also be located at or near reactive sites in the molecule since dilution alone does not cause an increase in fluorescence. The location of fluorescein near these sites would also explain the increase in *Kf* observed, since the antigen molecule would have to displace fluorescein before complex formation could occur.

To verify further that the degree of labeling of the two antitoxins was the same, the number of

fluorescein molecules per antitoxin molecule was experimentally determined. The second fluorescent antibody contained an average of 5.35 fluorescein molecules to 1 antitoxin molecule while the ratio was 5.4 to 1 for the third fluorescent antitoxin. This very similar labeling probably occurs because the amount of fluorescein used in both preparations was in excess of that required, the larger excess being adsorbed to a greater extent in the second preparation.

Fourth Fluorescent Antitoxin.—The presence of physically adsorbed fluorescein has been confirmed in the literature (11) for a different protein. By treatment with charcoal, a large percentage of this adsorbed material was removed. The fourth fluorescent antitoxin was made from the third preparation by shaking it with activated charcoal for 3 hours and filtering all traces of charcoal. This product showed a 50% decrease in overall fluorescence from the third preparation but only a 29.8% loss of *Lf*, which is comparable to the literature value. Immunochemically inert fluorescein had definitely been removed. Tests run on supernatants after flocculation showed no release of adsorbed fluorescein as had been noted previously. In addition, this material had good toxoid flocculating power, which had not been observed with the previous preparation, and typical precipitin curves could be obtained. The *Kf* with toxin had decreased to 8 minutes and toxoid *Kf* was in the range of 30 to 45 minutes. The toxoid reactivity loss from the original unlabeled antitoxin was 44% which is the same overall loss of toxin reactivity. An "unknown dilution" of toxin was assayed within 98.6% of its value as determined by *Lf* tests using a comparison standard toxin test run simultaneously. Toxoid could also be quantitated and tests on a series of dilutions gave a typical standard curve. Analysis showed an average of 5.25 molecules fluorescein to each molecule antitoxin. The charcoal adsorption evidently did not remove any significant quantity of chemically bound fluorescein. The activity and characteristics of this material show that it should be possible to prepare an antitoxin without the charcoal adsorption step provided excess adsorbed fluorescein can be avoided.

Fifth Fluorescent Antitoxin.—Reactants were used in proportions calculated to yield a product having a 1:1 ratio of fluorescein to protein. Examination showed that 100% of toxin and toxoid reactivity was retained in the process of synthesis and the *Kf* was 5 minutes. The molecular ratio of fluorescein to antitoxin was found to be 1.5 to 1. Supernatant examination after flocculation showed only a very slight liberation of bound fluorescein during reaction. The fast reaction and full retention of activity made this the best lot of antitoxin prepared at this point. The fluorescence level, however, was quite low, corresponding to the weak staining antibody discussed by Goldwasser (12).

Sixth Fluorescent Antitoxin.—The ratio of reactants was chosen to give a 5:1 fluorescein to protein product. One hundred per cent biological activity was retained for both toxin and toxoid. An examination of the supernatant after flocculation showed definite liberation of fluorescein during reaction, although this amount was much smaller than with the third antitoxin. The ratio of fluorescein to antitoxin in this preparation was 4.25 to 1. Flocculation fluorescence readings with this material read about 17% lower than those obtained with the fourth fluorescent antitoxin and the degree of labeling was 19% less, showing good correlation.

SUMMARY

Several fluorescent diphtheria antitoxins were prepared in order to determine the optimum conditions for obtaining a highly fluorescent antibody without denaturing the protein to the extent of losing significant flocculating power. The amount of fluorescein derivative used to prepare the fluorescent antitoxins was found to determine the *Kf* value and the amount of toxin or toxoid flocculating potency lost. There was an optimum amount of fluorescein which would couple chemically with the antitoxin, this value being an average of five molecules fluorescein to each antitoxin molecule. If amounts in excess of this were used, physical adsorption occurred which greatly decreased antitoxic potency. Antitoxoid potency was very easily destroyed by overlabeling. Some of the adsorbed fluorescein did not exhibit fluorescence until it was released by the reaction of the antitoxin with toxin or toxoid. It did not contribute to the fluorescence of the flocs and could be removed by competitive adsorption with activated charcoal.

The fluorescent antibody technique was shown to be applicable not only as a diagnostic or qualitative test but as a sensitive quantitative method of analysis as well. A comparison standard test, run simultaneously with the unknown, was more reliable than a standard curve procedure since varying conditions could be automatically controlled.

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Separation of Tabletted Mixtures of Barbiturates, Aspirin, Phenacetin, Caffeine, Codeine, and Quinine by Ion-Exchange Paper Chromatography

By HAROLD V. STREET and S. K. NIYOGI

The resolution of a mixture of tablet fragments containing amobarbital, acetylsalicylic acid, acetophenetidin (phenacetin), caffeine, codeine, and quinine into its constituent parts by application of chromatography on modified cellulose ion-exchange papers is described. Horizontal circular and ascending cylindrical chromatography is employed. A complete qualitative analysis takes about 4 hours.

MODIFIED CELLULOSE ion-exchange paper sheets were first used for chromatography by Knight (1, 2) who described their use for the separation of mixtures of amino acids and of mixtures of metallic cations. Street and Niyogi (3–5) have described the use of these modified cellulose papers for chromatographic and ionophoretic separations of various toxic compounds.

Because chromatography on ion-exchange papers with aqueous "solvents" gives rise to a very rapidly moving "solvent" front, it was thought the procedure might be useful for a rapid identification of some of the tablets which are commonly encountered in toxicological analysis. In this type of work, it is necessary for the toxicologist to carry out an analysis of any fragments of tablets which may be found in, say, stomach contents or cups and drinking glasses.

The types of tablets most frequently encountered in toxicological analysis are those containing barbiturates or salicylates. These tablets may, in addition, contain acetophenetidin (phenacetin), caffeine, codeine, and quinine. Using mixtures containing equal proportions of these compounds, it is a relatively easy matter to separate and identify the components by chromatography on ion-exchange paper. However, in many of the tablets, the proportion of one compound to another is such that swamping of the minor component occurs. For example, in tablet A,[1] the ratio of acetylsalicylic acid to quinine is 48 to 1, so that if a chloroform extract of the tablet is applied directly to the paper, the quinine cannot be seen because of the presence of such a relatively large amount of salicylate which spreads over a large area of the paper and obliterates the quinine spot. If the amount applied is reduced so that the salicylic acid spot is of "normal" size, then the amount of quinine is too small to be detected.

When acetylsalicylic acid and quinine are present in equal amounts, no difficulty is encountered. It follows, therefore, that in order to detect the presence of quinine in tablet A, some preliminary differentiation procedure must be used, such as extraction with alkali to remove acetylsalicylic acid. This procedure will, of course, hydrolyze the aspirin to salicylic acid which, however, is easily recognized on the chromatogram by its bright blue fluorescence in 254 mμ light.

It has been found that by extraction of tablet fragments with chloroform and then extracting this chloroform with alkali, the two groups so obtained, namely neutral and acidic, can be applied to an anion-exchange paper and the various components identified. Quinine and codeine can be identified in the residue by chromatography on a cation-exchange paper. Horizontal circular chromatography as described by Kawerau (6) has been used with hand-cut, slotted, Whatman diethylaminoethyl cellulose ion-exchange paper, together with a simultaneous ascending "run" on Whatman cellulose citrate ion-exchange paper. The following account is a description of the procedures carried out for the identification of the compounds referred to above.

It is important to note that the work described here refers to modified cellulose ion-exchange paper and not to paper impregnated with ion-exchange resins.

EXPERIMENTAL

Materials and Reagents

Horizontal circular chromatography apparatus manufactured by Shandon Scientific Co., Cromwell Place, London, England. Whatman DE20 anion-exchange modified cellulose paper; the sheets were cut into circular disks of 26 cm. diameter, slots were cut as described by Kawerau (6). Whatman CT30 cation-exchange modified cellulose paper.

Received August 14, 1961, from the Department of Forensic Medicine, University of Edinburgh, Scotland.
Accepted for publication October 16, 1961.

[1] Anadin (International Chemical Co., Ltd.) tablets contain in each: aspirin, 4 gr.; phenacetin, 2 gr.; caffeine, 1/4 gr.; and quinine sulfate, 1/12 gr.

Ultraviolet lamp emitting 254 mμ radiation. Chloroform. Sodium hydroxide, 2.5% (w/v) and 10% (w/v) aqueous solutions. Hydrochloric acid, 5 *N* solution. Ammonia, 0.2 *N* and concentrated solutions. EDTA, 0.1 *M* solution of the disodium salt of ethylenediaminetetra acetic acid. Iodoplatinate reagent; mix together 5 ml. of 5% (w/v) platinic chloride, 45 ml. of 10% (w/v) potassium iodide, and 100 ml. of water.

Procedure

Extraction.—A mixture of one-fifth of a tablet of tablet A, one-fifth of a compound tablet of codeine B.P., and 10 mg. of amobarbital was ground up in a mortar with 2 ml. of chloroform, and filtered. The mortar was rinsed with a further 2 ml. of chloroform which was poured through the filter. The filtrate was shaken vigorously with 0.5 ml. of 2.5% sodium hydroxide solution and the mixture centrifuged. The upper alkaline layer (solution I) was removed and set aside for chromatography. The chloroform layer was washed first with 4 ml. of water and then with 0.5 ml. of 5 *N* hydrochloric acid solution, and the mixture was centrifuged. These washings were discarded. The washed chloroform layer containing acetophenetidin and caffeine was set aside for chromatography (solution II).

The residue of the tablet fragments, after chloroform extraction, was shaken with 10 ml. of 10% sodium hydroxide solution and filtered. The filtrate was extracted by shaking with 10 ml. of chloroform. A 1-ml. portion of the separated chloroform layer was taken to dryness and redissolved in a drop or two of chloroform. This solution was used for chromatography (solution III).

Chromatography.—Solutions I and II were applied to two separate sectors of a circular sheet of Whatman DE20 ion-exchange paper. Chromatography was carried out in 0.2 *N* ammonia solution for 105 min. and the paper examined in 254 mμ light. The results of such a run are illustrated in Figs. 1 and 2.

Solution III was subjected to ascending chromatography on Whatman CT30 ion-exchange paper in 0.1 *M* EDTA for 18 minutes. The paper was examined, while wet, in 254 mμ light; it was then dried and stained with the iodoplatinate reagent to reveal the positions of the alkaloids. The results of this procedure are shown in Fig. 3.

DISCUSSION

The results show that with the DE20 paper, using dilute ammonia as solvent, barbiturate and salicylate can be separated in the alkali extract. Identification of the particular barbiturate can be carried out by the methods of Street (7, 8). Acetophenetidin and caffeine, both of which are present in the residual chloroform solution after alkali and acid extraction, can also be resolved on DE20 paper. Because there is a large amount of salicylate present in the tablet fragments, this compound is also seen in the "neutral" fraction. It does not interfere and is easily distinguished by its bright blue fluorescence in 254 mμ light.

It is interesting to note that in spite of the presence of a relatively large amount of acetophenetidin, this does not interfere with the resolution of acetophenetidin and caffeine in the "neutral" fraction. This

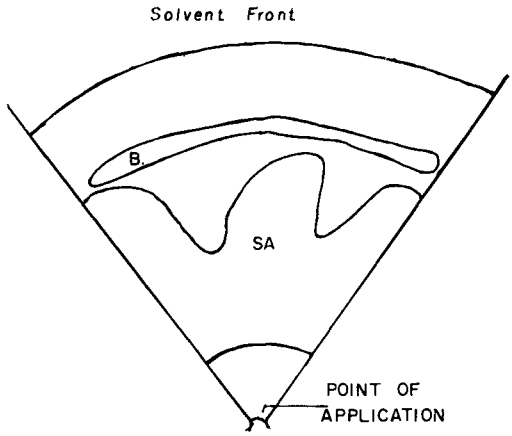


Fig. 1.—Horizontal circular chromatography of solution I on diethylaminoethyl cellulose ion-exchange paper in 0.2 *N* ammonia. Time of run, 105 min. SA, salicylate (blue fluorescent area); B, barbiturate (dark-purple absorbing area). Compounds revealed by inspection in 254 mμ light.

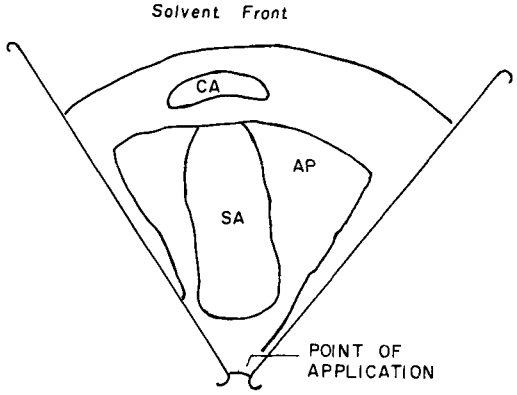


Fig. 2.—Horizontal circular chromatography of solution II on diethylaminoethyl cellulose ion-exchange paper in 0.2 *N* ammonia. Time of run, 105 min. SA, salicylate (blue fluorescent area); AP, acetophenetidin (dark-purple absorbing area); CA, caffeine (dark-purple absorbing area). Compounds revealed by inspection in 254 mμ light.

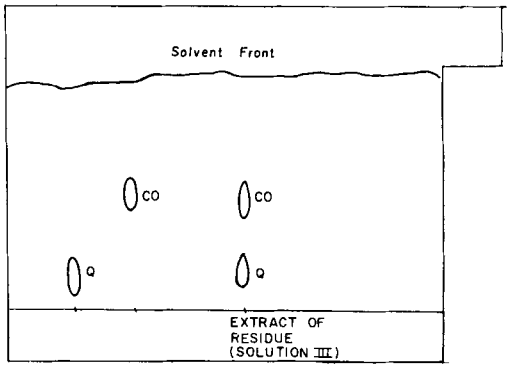


Fig. 3.—Ascending cylindrical chromatography of solution III on cellulose citrate ion-exchange paper in 0.1 *M* EDTA. Time of run, 18 minutes. Q, quinine; CO, codeine. Compounds revealed by dipping the paper in the iodoplatinate reagent.

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Codeine and quinine are extracted by chloroform from the residue of tablet fragments after addition of alkali. These two alkaloids are clearly resolved on CT30 paper in about 20 minutes. Some quinine is also present in the initial chloroform extract of the tablets but is removed from the "neutral" fraction by washing with dilute acid.

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Diffusion of Sodium Salicylate and Salicylic Acid within Hydrophilic Ointments

Measurement with a New Diffusion Cell

By J. A. WOOD†, L. WAIT RISING, and NATHAN A. HALL

A cell has been designed to measure diffusion of medicaments within dermatologic vehicles to eliminate the influence of an external medium which differs from the vehicle. The cell consisted of two Lucite plastic compartments separated by a cellophane membrane. A medium containing a medicament was placed in one compartment and diffusion occurred into the other compartment which contained the same vehicle without the medication. The cell was used in a study of the diffusion of sodium salicylate and salicylic acid in various components of hydrophilic ointments made with three nonionic surface-active agents: Tween 40, Atlas G-7596-J, and Brij 35.

IN VITRO investigations of the efficiency of medicated dermatologic vehicles have usually utilized a system in which the drug diffuses from the vehicle into a dissimilar medium. Events which occur in this external medium provide the data for the evaluation of the vehicle. Workers in this field have noted the desirability for additional knowledge of diffusion within the base and its physicochemical properties (1–3). It is possible that in many reported experiments the results obtained have been more dependent upon the rate of diffusion in the external medium than upon the rate of diffusion in the vehicle being tested. Fuller, *et al.* (1), suggested that the rate of absorption of a drug from a locally applied vehicle will depend on the rate of the slowest of the processes involved in the transfer of medicament to the tissue fluids. Thus, *in vitro*, the limiting influence could occur in the external medium, at the interface, or in the preparation containing the medicament. One significant physical property is diffusion of drug molecules within the vehicle.

A cell has been developed which makes possible the measurement of diffusion of a drug within its vehicle and it has been applied to diffusion measurements of salicylic acid and sodium salicylate in hydrophilic ointments prepared with three different emulsifiers. Studies involved media of increasing complexity from simple aqueous solutions to fluid emulsions to semisolid hydrophilic ointments.

Received October 14, 1961, from the College of Pharmacy, University of Washington, Seattle 5.
Accepted for publication November 15, 1961.
Abstracted in part from a thesis presented by J. A. Wood to the Graduate School, University of Washington, in partial fulfillment of the requirements for the Ph.D. degree.
† Present address: College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

EXPERIMENTAL

The Diffusion Cell.—Since free diffusion studies in liquid media require rather elaborate methods to avoid boundary disturbances due to the effect of convection currents, vibration, and gross movement of the apparatus, Geddes (4) suggested separation of the phases by a porous diaphragm to eliminate practically all boundary disturbance. Preliminary studies indicated that methods which brought

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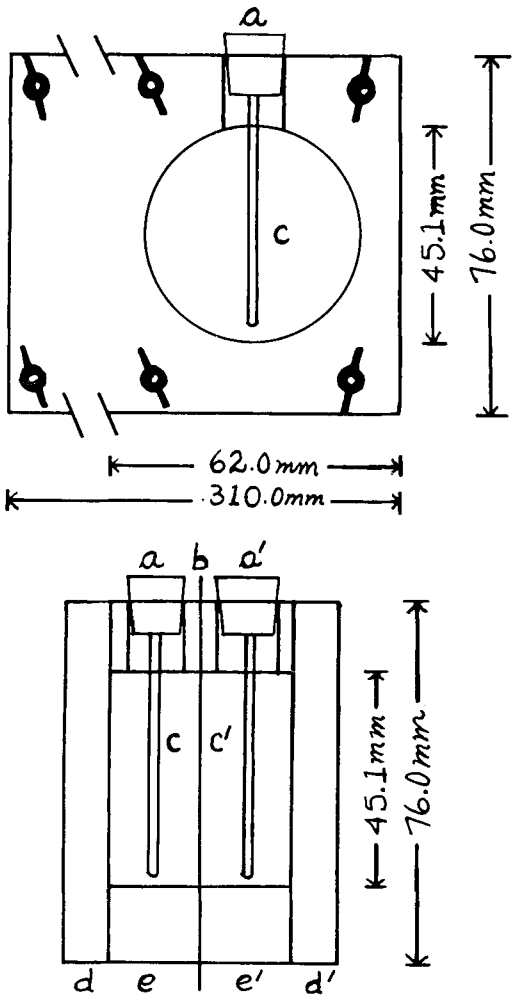


Fig. 1.—One section of the five-cell Lucite diffusion unit showing side view (top) and end view (bottom). d,d' is 3/8 inch clear Lucite and e,e' is 3/4 inch clear Lucite.

medicated and nonmedicated portions of ointment (semisolid) into contact were suitable for visual observation of diffusion, but not suitable for removing samples for analysis. In order, therefore, to maintain stable boundary conditions in the liquid vehicles, and to permit separation of the two parts of the semisolid diffusion system, an intervening cellophane membrane was used. The vehicle was thus separated into two aliquots, one on each side of the membrane. At the beginning of each experiment, only one aliquot contained medicament. Samples could be removed at different time intervals for analysis to give the diffusion rate.

The diffusion apparatus consisted of a Lucite unit containing five diffusion cell compartments. Each cell was cylindrical in shape (Fig. 1) with an opening (12.5 mm. diameter) (a,a') in each section of the cylinder (c,c') for filling the cell and subsequently removing its contents. Stirring rods were inserted into rubber stoppers and installed in the openings (a,a'), serving both as stirring devices and as closures for the cells. To assemble the apparatus, the cellophane[1] (b) was placed at the center of the four components (Fig. 1, end view), and the unit was assembled by tightening the wing nuts on the bolts holding the unit together. The assembled unit, ready for use, was photographed for Fig. 2. The internal dimensions were such that when 28.5 ml. of liquid was placed in each side of the cell (c,c') and stirring rods positioned, the cell was completely filled to the bottom of the openings and the membrane (16 sq. cm.) was completely covered.

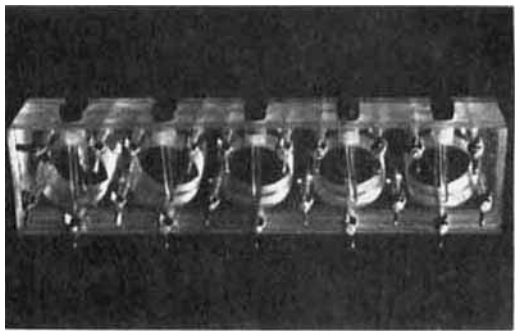


Fig. 2.—The assembled Lucite plastic diffusion cell unit.

For semisolid media the diffusion cell differed in only one respect from that used for the liquid systems; namely, the center components were constructed from 3/8 inch Lucite. The depth of each half of the cell was thus 3/4 inch instead of 3/4 inch. Stirring rods were of no use, but openings to each cell compartment (6 mm. in diameter) were provided to permit the escape of ointment from the slightly overfilled cells. Slight overfilling with consequent pressure exerted on complete assembly was utilized to assure intimate contact between the ointment contained in each cell compartment and the cellophane membrane. The shallow-depth cell was used to facilitate chemical analysis because of the relatively short depth of penetration of medicament into the receptor ointment.

Diffusion Media.—Diffusion experiments were conducted on media which were variations of components of the terminal medium. The terminal medium was a modified form of hydrophilic ointment U.S.P. XV (5) of the following formula

	Quantity, Gm.
Surface-active agent[2]	5.0
Propylene glycol	12.0
Liquid petrolatum	25.0
Stearyl alcohol	25.0
Preserved water C.F. (6)	33.0

The simplest medium was preserved water C.F. (6), and other components of the formula were added to give media of increasing complexity until the hydrophilic ointment was formed. The quantities of ingredients in the simpler media were in the same ratio as in the terminal media. Three series of experiments were performed, differing only in the surface-active agent used as the emulsifier. Com-

[1] The humectant was removed by continuous rinsing with distilled water followed by rinsing with the diffusion medium.

[2] Tween 40 (polyoxyethylene sorbitan monopalmitate), Atlas G-7596-J (polyoxyethylene sorbitan monolaurate), or Brij 35 (polyoxyethylene lauryl ether), all supplied by Atlas Powder Co., Canada, Ltd., Brantford.

TABLE I.—RATE OF DIFFUSION OF SODIUM SALICYLATE AND SALICYLIC ACID AND THE VISCOSITY OF THE DIFFUSION MEDIA

Sodium Salicylate Diffusion			Salicylic Acid Diffusion		
Diffusion Designation[a]	Diffusion, mg./cm.²/hr.	Viscosity of Diffusion Media, cps.	Diffusion Designation[a]	Diffusion, mg./cm.²/hr.	Viscosity of Diffusion Media, cps.
A	36.1	0.92	a	0.517	0.92
B	17.9	2.19	b	0.221	2.19
C1	27.5	2.08	c1	0.107	2.08
D1	15.0	3.67	d1	0.075	3.67
E1	5.55	22.5	e1	0.050	20.3
F1	0.29	26,000	f1	0.0020	26,000
C2	27.6	2.20	c2	0.083	2.15
D2	13.7	3.40	d2	0.068	3.49
E2	5.65	20.4	e2	0.067	20.7
F2	0.34	24,000	f2	0.0023	24,000
C3	24.1	3.59	c3	0.058	3.60
D3	12.7	5.60	d3	0.050	5.80
E3	5.40	30.0	e3	0.034	30
F3	0.25	30,000	f3	0.0018	30,000

[a] Upper case letters indicate sodium salicylate and lower case salicylic acid. Arabic numerals indicate the emulsifier used in each series: 1, Tween 40; 2, Atlas G-7596-J; 3, Brij 35; Aa, preserved water C.F.; Bb, same as A plus propylene glycol; Cc, same as A plus emulsifier; Dd, same as B plus emulsifier; Ee, same as D plus liquid petrolatum; Ff, same as E plus stearyl alcohol, a hydrophilic ointment.

ponents of each medium are indicated in Table I which presents the results.

Medicaments.—Sodium salicylate U.S.P. and salicylic acid U.S.P. were incorporated into the diffusion media to form the medicated preparations. Salicylic acid, a commonly used agent in dermatology, was selected for its low aqueous and higher lipid solubility and sodium salicylate for its reverse solubility characteristics. The concentration of medicament was arbitrarily set at 12% for sodium salicylate, and because of its low water-solubility, the salicylic acid concentration was 0.2% in the fluid preparations and 1% in the semisolid medicated preparations.

The Diffusion Procedure.—The diffusion procedure for liquid preparations was as follows: The cell unit was immersed almost to the top in a constant-temperature water bath at 30°. Exactly 28.5 ml. of diffusion medium was transferred to the receptor side of the first cell and the stopper and stirring rod were positioned without pressure. Exactly 28.5 ml. of medicated preparation were transferred to the donor side of the first cell, the time of completion noted, and the stopper and stirring rod positioned without pressure. The other cells were filled in succession in the same manner. At 15-minute intervals for sodium salicylate and 30-minute intervals for salicylic acid, the solution in each side of each cell was stirred 10 times. Sixty minutes after the sodium salicylate diffusion had started (120 minutes for salicylic acid), the two solutions were removed completely from each cell (donor and receptor), placed in appropriately labeled flasks, and reserved for analysis and examination. On completion of the analysis of the five receptor solutions, they were combined and the five donor solutions were combined. These two solutions, donor and receptor, were then subjected to determination of density, pH, surface tension, and viscosity.

For diffusion experiments involving the semisolid terminal media, the diffusion cell chambers were filled by the use of a spatula as the unit was assembled. Care was exercised to avoid air spaces in the material and between the ointment and the cellophane. After an appropriate diffusion period in an incubator at 30° (18 hours for sodium salicylate and 60 hours for salicylic acid), the preparations were carefully removed, weighed, and placed in suitable containers for analysis and examination.

Physical Properties.—Certain physical data were routinely compiled for each diffusion study. These were density, pH, surface tension, and viscosity. The solubility of the medicament in the vehicle (7) was also determined. The Brookfield UL adapter for use with the Brookfield Synchro-Lectric viscometer, Model LVT, for the measurement of ultra low viscosities, was used to determine the viscosities of all of the fluid preparations. Corrected apparent viscosities were calculated for the emulsified media according to Bowles' method (8). Approximate viscosities for the semisolid preparations were determined using the model RVT viscometer. Only viscosity values are recorded in the results since it was the only property paralleling the differences in diffusion rates.

Method of Analysis.—Salicylate concentrations were determined colorimetrically by measurement of color produced by the reaction of ferric chloride with salicylates. The readings were taken at 525 mμ with a Beckman model B spectrophotometer. The emulsion preparations could not be assayed by direct dilution because of the insoluble components. Preparations emulsified with Tween 40 and G-7596-J were broken down by heating with 2 *N* sodium hydroxide, thus permitting separation of the aqueous phase. Since Brij 35 was stable to alkaline hydrolysis, the aqueous phase was separated by saturation with sodium chloride.

RESULTS AND DISCUSSION

The rates of diffusion of sodium salicylate and salicylic acid in the various media and the viscosities of these media are shown in Table I. The rates of diffusion are compared in Fig. 3.

The results of the investigation showed, as expected, that in water and solutions of propylene glycol in water, the rate of diffusion was very closely related to the viscosity of the medium. When

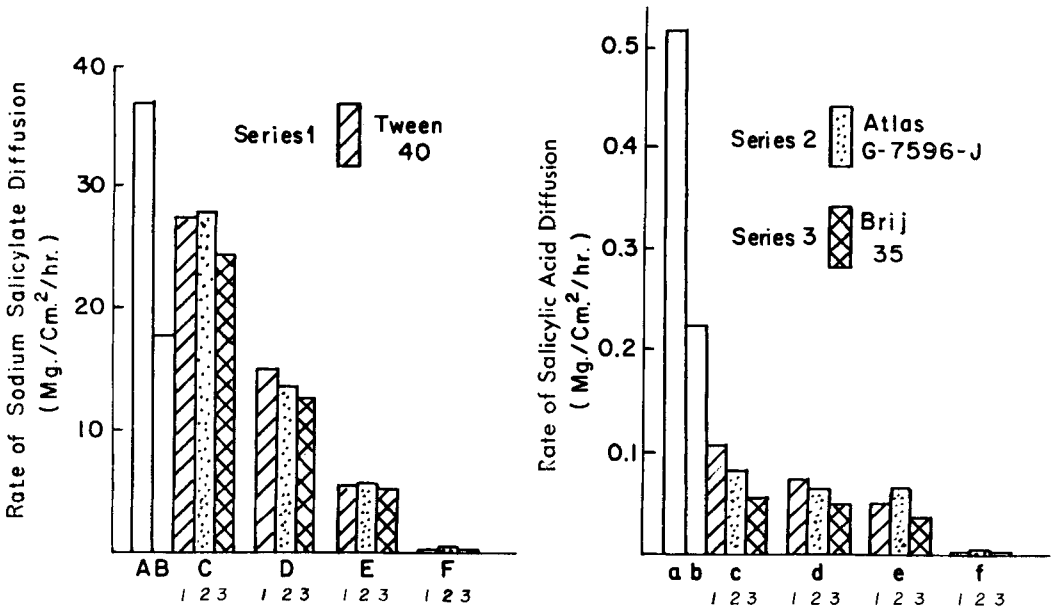


Fig. 3.—The effect on sodium salicylate and salicylic acid diffusion of adding each formula component in the three series and relating these to diffusion in Aa and Bb. Aa = Preserved water; Bb = A + propylene glycol; Cc = A + surfactant; Dd = C + propylene glycol; Ee = D + liquid petrolatum; Ff = E + stearyl alcohol.

a surface-active agent was added, the change in viscosity was similar to the change produced by the propylene glycol, but the reduction in the rate of diffusion resulting was only approximately half the reduction caused by propylene glycol. Since aqueous solutions of surface-active agents form micelles, these could be responsible for a measured viscosity which has less effect on a diffusing molecule or ion than the viscosity due to a substance like propylene glycol which does not form micelles. The bulk viscosity of propylene glycol solution, measured with the viscometer, approximates a microscopic viscosity. In the surface-active agent solutions containing micelles, a much greater difference exists between the bulk and microscopic viscosities. The fact that the diffusion effects of propylene glycol and a surface-active agent are additive only to a low degree also is probably related to the same phenomenon.

In contrast to the sodium salicylate diffusion system, once the surface-active agent is added to the salicylic acid diffusion system there is a marked reduction in the diffusion rate and only relatively small changes result from the additon of propylene glycol and liquid petrolatum. Since it can be shown that salicylic acid is solubilized by the micelles of surface-active agents, the presence of micelles in which salicylic acid is preferentially soluble would retard the diffusion process.

All components in each series which increased viscosity caused a reduction in the rate of diffusion from that in simple aqueous solution. The largest reduction was due to stearyl alcohol which transformed the media from liquid to semisolid with a consequent very large increase in viscosity. The effect due to differences in the surface-active agents were slight (see Fig. 3). It may be noted, however, that all preparations containing Brij 35 were somewhat more viscous than those containing the other two emulsifiers and, generally speaking, the rate of diffusion in media containing Brij 35 was slightly lower.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product I

Reproducibilities of Assay and Drug Recovery from Dosage Forms

By EDWARD R. GARRETT†

Studies have been conducted on the variation of fill weight and drug content among units of hard filled capsules, soft elastic capsules, and compressed tablets. The cases in point were exploratory dosage forms of the antibiotic fumagillin. Consideration of the dosage form and the excipients dictated the methodology. Statistical treatment of the data has permitted conclusions as to optimum amounts of solvent for the separation of the active ingredient, the number of dosage units necessary to assay within a desired confidence limit, the reproducibility of fill weight among units, and the homogeneity of the fill mix. The evaluation of the variability among the data also permitted the most economic utilization of assay facilities in stability studies.

A BASIC FUNCTION of the control laboratories in the pharmaceutical industry is to assay and control the final product so that it conforms to those specifications which have been established to insure that (*a*) the composition is and remains as specified, (*b*) the pharmaceutical utility is retained, and (*c*) the pharmaceutical elegance of color, taste, and form persists; all for the duration of the pharmaceutical lifetime of such a product.

Routine assay procedures for final products must be carefully designed so that they are simple, efficient, and economical. These considerations necessitate the application of statistical techniques for the proper evaluation of assay procedures and the determination of errors of assay. Thus, the degree of confidence in product composition, stability estimates, and biological potency may be ascertained.

This paper considers the fundamental problems of recovery of drug from the final pharmaceutical dosage form, the number of such dosage forms to be used in assay, and the variability in content that may be encountered among units of the same dosage form. Dosage forms considered are hard filled capsules (HFC), soft elastic capsules (SEC), and compressed tablets (CT). The procedures of the simple statistics used are easily followed from many of the fine texts available (1).

The particular drug considered in these studies was the antibiotic fumagillin (2–4) in exploratory preparations of HFC, SEC, and CT. The initial reason for this comparative study was to establish the minimum and most economical procedures for the evaluation of the drug's stability in its final dosage form.

Received September 5, 1961, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication October 18, 1961.
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EXPERIMENTAL

The ultraviolet spectrophotometric absorbance of fumagillin at 351 mμ in 95% ethanol-water was chosen as the method of determining fumagillin content since these values have been correlated with biological activity (2). Photolytic, oxidative, and thermal degradation of fumagillin in solution and as crystalline material diminishes the absorbance at 351 mμ (2–4). The Beckman ultraviolet spectrophotometer, model DU, was used.

The procedures devised for the spectrophotometric determination of fumagillin content follow.

Hard Filled Capsules.—The theoretical composition of the HFC was 12 mg. fumagillin, 10 mg. ascorbic acid, 226 mg. lactose, 1.3 mg. calcium stearate, with a fill weight of 259 mg. An initial study was conducted on 10 capsules where each capsule was weighed on an analytical balance and subsequently emptied into a beaker by tapping or gouging with the end of a fine spatula. The capsule was washed twice with 95% ethanol, the spatula was rinsed with

TABLE I.—FILL WEIGHT AND SPECTROPHOTOMETRIC ASSAY OF FUMAGILLIN CONTENTS OF HARD FILLED CAPSULES

Capsule No.[a]	Wt. of Fill (*W*)	Absorbance (*A*)[b] at 351 mμ	*A*/*W*
1	0.2613	0.454	1.7375
2	0.2597	0.410	1.5787
3	0.2562	0.379	1.4793
4	0.2576	0.380	1.4752
5	0.2572	0.323	1.2558
6	0.2535	0.377	1.4872
7	0.2576	0.373	1.4480
8	0.2671	0.517	1.9356
9	0.2588	0.477	1.8431
10	0.2579	0.477	1.8496

[a] A 10-ml. quantity of 95% ethanol was used to extract fumagillin from each of capsules 1–7; 50 ml. from each of capsules 8–10. [b] Measured at 4×10^{-4} capsule contents per ml. final solution.

solvent, and all washings and contents were collected so that a total of 10 ml. solvent to extract was used in seven cases and a total of 50 ml. was used to extract in the other three cases. The material was allowed to settle and the contents of the beaker were decanted through No. 2 Whatman filter paper. The residue was washed with two 2-ml. portions of ethanol, filtered, and the filtrate was made up to 100 ml. in a volumetric flask with 95% ethanol. A 10-ml. aliquot of this solution was made up to 250 ml. The result was that there was 4×10^{-4} of the original capsule per ml. of final solution. This concentration could be spectrophotometrically read in the appropriate absorbance range (*ca.* 0.5) at 351 mμ. The weight of the empty capsule was determined after drying at room temperature so that the net weight of fill could be obtained. These data are given in Table I.

A similar experiment was conducted on each of six HFC except that the volume of 95% ethanol used to extract the fumagillin was varied. See Table II.

TABLE II.—ABSORBANCES OF HFC FUMAGILLIN AT 351 mμ EXTRACTED WITH VARYING AMOUNTS OF 95% ETHANOL

Capsule No.	95% Ethanol Used in Extraction, ml.	Absorbance at 351 mμ[a]
11	10	0.418
12	20	0.442
13	30	0.430
14	40	0.479
15	50	0.484
16	75	0.493
17	100	0.489

[a] At 4×10^{-4} capsule contents per ml. of final solution.

Soft Elastic Capsules.—The theoretical composition of the SEC was 12 mg. fumagillin, 10 mg. ascorbic acid, 160 mg. peanut oil, 5.27 mg. Cotoflake, with a fill weight of 196 mg. Each of 10 capsules was weighed on an analytical balance and cut almost in half over a beaker with a sharp, clean razor blade. Extreme care was taken to get no oil on the fingers. The entire capsule was dropped into the skellysolve B rinsings of the razor blade edge (*ca.* 15 ml.) and swirled to remove the contents. The SEC shell was removed and rinsed with a few ml. of fresh skellysolve B which was then added to the contents of the beaker. The shell was dried in a hood at room temperature and re-weighed to obtain the net weight of the fill. The skellysolve B was evaporated from the beaker in the hood at room temperature, and 50 or 100 ml. of 95% ethanol was added to attempt dissolution. Subsequent filtration and preparation of the solution for spectrophotometric assay was the same as for that previously given for the HFC with 4×10^{-4} of the original capsule contents per ml. of final solution. The data are given in Table III.

Compressed Tablets.—The theoretical composition of the CT was 12 mg. fumagillin, 10 mg. ascorbic acid, 226 mg. lactose, 9.8 mg. sucrose, 6.5 mg. starch, 2.3 mg. calcium stearate, with a weight of 277 mg. Each tablet was weighed on an analytical balance and dropped into 95% ethanol. The tablet was extracted with various amounts of ethanol as specified in Table IV. Subsequent filtration and preparation of the solution for spectrophotometric assay was the same as for that previously given for the HFC with 4×10^{-4} of the original tablet per ml. of final solution. The data are given in Table IV.

TABLE III.—FILL WEIGHT AND SPECTROPHOTOMETRIC ASSAY OF FUMAGILLIN CONTENTS OF SOFT ELASTIC CAPSULES

Capsule No.[a]	Wt. of Fill (W)	Absorbance (A)[b] at 351 mμ	A/W
1	0.2011	0.542	2.7001
2	0.2030	0.533	2.6256
3	0.1929	0.510	2.6439
4	0.1698[c]	0.447	2.6325
5	0.2012	0.556	2.7634
6	0.2120	0.591	2.7877
7	0.1993	0.507	2.5439
8	0.2043	0.552	2.7019
9	0.1979	0.608	3.0723
10	0.2024	0.575	2.8409

[a] A 50-ml. quantity of 95% ethanol was used to extract fumagillin from the contents of each of capsules 1–5; 100 ml. from contents of each of capsules 6–10. [b] At 4×10^{-4} capsule contents per ml. final solution. [c] This datum is low; it may be queried if this is a maverick or if it has a real probability of occurrence. See text.

TABLE IV.—COMPRESSED TABLET WEIGHT AND SPECTROPHOTOMETRIC ASSAY OF FUMAGILLIN CONTENT

Tablet No.[a]	Wt. of Tablet (W)	Absorbance (A)[b] at 351 mμ	A/W
1	0.2737	0.460	1.680
2	0.2832	0.472	1.667
3	0.2795	0.464	1.660
4	0.2816	0.475	1.687
5	0.2761	0.470	1.702
6	0.2808	0.475	1.692
7	0.2778	0.475	1.710
8	0.2760	0.472	1.710
9	0.2799	0.481	1.718

[a] A 50-ml. quantity of 95% ethanol was used to extract fumagillin from each of tablets 1–3; 75 ml. from each of tablets 4–6; and 100 ml. from each of tablets 7–9. [b] At 4×10^{-4} tablet per ml. final solution.

TABLE V.—ANALYSIS OF VARIANCE OF ABSORBANCE AT 351 mμ PER GM. TABLET WEIGHT (A/W) FOR VARYING AMOUNTS OF ETHANOL EXTRACTION[a]

Source	Sum of Squares	Degrees of Freedom	Variance
Among extractions	0.0030481	2	0.001524
Within extractions	0.0002208	6	0.0000368
Total	0.0032689	8	0.0004086

[a] Quantities of 50, 75, and 100 ml.

CALCULATIONS AND RESULTS

The data of Table I indicate a large difference in measured absorbance between HF capsules 1–7 (average $A/W = \bar{x}_1 = 1.4945$ for $n_1 = 7$) extracted with 10 ml. 95% ethanol and capsules 8–10 (average $A/W = \bar{x}_2 = 1.8761$ for $n_2 = 3$) extracted with 50 ml. 95% ethanol. Application of the t test confirms this difference. The calculated t is $(\bar{x}_2 - \bar{x}_1)/\sigma \cdot \sqrt{n_1 n_2/(n_1 + n_2)} = 3.73$ at 8 degrees of freedom. This exceeds the critical value from the t tables, i.e., 2.31 for 5% probability of no difference between the extraction procedures. It is thus necessary to

TABLE VI.—SUMMARY OF REPRODUCIBILITY OF PHARMACEUTICAL PREPARATIONS OF FUMAGILLIN

	HFC	SEC	CT
Fill wt. and 95% confidence limits of one tablet or capsule ($\bar{x} \pm t\sigma$)[a]	259 ± 8 mg. (259)	202 ± 12 mg. (196)[c]	279 ± 7 mg. (277)
% Deviation of single unit from mean, 100 ($\sigma/\bar{x}$)	1.39%	2.56%[c]	1.09%
Absorbance at 351 mμ and 95% confidence limits ($\bar{x} \pm t\sigma$)	0.483 ± 0.017[b]	0.553 ± 0.080	0.472 ± 0.015
% Deviation of single absorbance from mean, 100 ($\sigma/\bar{x}$)	1.39%[b]	6.21%	1.34%

[a] Theoretical fill weight given in parentheses. [b] Includes only those HFC extracted with 40 or more ml. of 95% ethanol. [c] SEC No. 4 in Table III is excluded.

determine the minimum amount of 95% ethanol necessary for complete extraction of fumagillin from the contents of the hard filled capsules. These data are reported in Table II and readily show that 40 or more ml. of 95% ethanol is necessary for relatively complete extraction of fumagillin from one capsule.

From the data of Table III we have, for capsules numbered 1 through 5, the average $A/W = \bar{x}_1 = 2.6731$ for $n_1 = 5$, and for capsules numbered 6 through 10, the average $A/W = x_2 = 2.7893$ for $n_2 = 5$. The calculated t for 8 degrees of freedom does not exceed the Table's value of t. It is concluded that either 50 or 100 ml. of 95% ethanol will give satisfactory extraction of fumagillin from the contents of each soft elastic capsule.

The data of Table IV for compressed tablets were obtained from extractions with 50, 75, and 100 ml. of 95% ethanol. An analysis of variance of these data calculated as absorbance per unit weight of fill, A/W, is given in Table V. The calculated F value for the ratio of variance among extractions to variance within extractions is 41 for 2 and 6 degrees of freedom. The F value at the 1% level from the tables is 10.9. It can be concluded that the variation among volumes of extraction is highly significant with respect to the variation within extraction by the same volume of 95% ethanol. This does not necessarily mean that the use of 100 ml. rather than 50 ml. of ethanol introduces a large error in this case, only that our methods of measurement are precise enough to evaluate the differences. In fact, the difference in extractability is extremely small, percentage wise, only 2.5% between the mean A/W values for 50 ml. and 100 ml. ethanol extractions (see Table IV).

The per cent standard deviation, $100 \times \sigma/\sqrt{(A/W)}$, among all the tablets is 1.2%. This includes the possible heterogeneity of fumagillin in the tablet mix, the procedural error, and the variation among volumes of ethanol used in extraction.

The reproducibility of the fill weight and the absorbance of extracted fumagillin in each of the HFC, SEC, and CT preparations of fumagillin are summarized in Table VI. The 1.4% probable deviation in absorbance from capsule to capsule, HFC, is the same as the estimated probable deviation in fill weight, which indicates that the fill may be of homogeneous fumagillin content as a first approximation provided that sufficient volumes of extracting solvent are used. This is confirmed by the consistency of the absorbances of capsules 14 through 17 in Table II.

DISCUSSION

These investigations and the simple statistics involved contribute a great deal of information. They permit an evaluation of the best methodology for the assay of the pertinent component of a finished pharmaceutical.

The fundamental step is to separate the drug from the assay-interfering substances of the final product and prepare it appropriately for assay. In the cases of the HFC and SEC preparations, this involved a physical manipulation of each unit. Spectrophotometric assay procedures necessitated the preparation of homogeneous solutions. Alcoholic solution with subsequent filtration was a satisfactory method to separate the fumagillin from the excipients of the HFC fill and the tablets.

The oil base and nature of the SEC demanded the use of an oil-miscible solvent for separation and skellysolve B was an appropriate choice.

Simple statistical evaluations permitted the choice of optimum amounts of solvent to use in the assay procedures.

The single tablet or capsule technique clearly showed a high reproducibility of fumagillin content in a single HFC and tablet (Table VI).

The determination of variations in fumagillin content per unit dosage form, fill weight, and the ratio of these two values demonstrated that modern pharmaceutical processing may produce a high degree of homogeneity in the prefill mix and a high degree of reproducibility per unit HFC and tablet.

The knowledge of a standard deviation, σ, permits determination of the number, n, of units to be used to give a control value within the error range desired, i.e. $\sigma/\sqrt{n}$. For example, if 2% error were acceptable, the absorbance values from nine soft elastic capsules should be averaged which would reduce the 6% standard deviation of a single capsule cited in Table VI. This would be a valid procedure if the variation among capsules can be attributed solely to the error in the assay of a single capsule.

The greatest limitation on elegant stability studies on finished pharmaceutical products is the expense of assay. Certainly in the case of these HFC and CT preparations, assay of single unweighed units at timed intervals under accelerated or storage conditions would minimize cost and yet give excellent stability data.

The evaluation of the data for the SEC show that this preparation is not as suitable. The variation in reproducibility of fill weight is relatively high 2.6% (Table VI), and the total fumagillin content

varies even more (6.2%), even with the exclusion of the anomalous capsule No. 4 of low fill weight. Capsule No. 4 may either have a reasonable probability of occurrence or may be a maverick, possibly due to either improper weighing or being the first or last capsule prepared from the lot. Good control practice would demand repeat assays to clarify this.

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(1) *a* Brownlee, K. A., "Industrial Experimentation," Chemical Publishing Co., Inc., Brooklyn, New York, 1953; *b* Bennett, C. A., and Franklin, N. L., "Statistical Analysis in Chemistry and the Chemical Industry," John Wiley & Sons, Inc., New York, N. Y., 1954; *c* Davies, O. L., "Statistical Methods in Research and Production," 3rd ed., Hafner Publishing Co., New York, N. Y., 1957.
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Preparation and Properties of New Gastric Antacids V

Aluminum-Magnesium Hydroxide Dried Gels

By STEWART M. BEEKMAN

The preparation of nine aluminum-magnesium hydroxide gels in the Al:Mg atomic ratio range of 2:1, 1:1, and 0.5:1 by a new process is described. The determination of the antacid activity by the modified *in vitro* method of Holbert, Noble, and Grote shows that dried gels stabilized with glycine or sorbitol have antacid characteristics similar to clinically proved liquid aluminum-magnesium hydroxide gel. Blends of the various components separately dried are shown to be relatively ineffective by comparison. The new blends of old antacids are predominantly simple mixtures and not new compounds. Unstabilized dried gels show a substantial aging effect.

In 1944 Rossett and Flexner (1) concluded that mixtures of milk of magnesia with aluminum hydroxide gel were more effective gastric antacids than aluminum hydroxide gel alone. They reached these conclusions as a result of *in vivo* experiments on humans together with extensive clinical experience. They suggested smaller and less frequent doses of the mixtures. They pointed out the avoidance of the undesirable high initial pH rise from the milk of magnesia alone, and the offsetting of the constipating effect of aluminum hydroxide. The outstanding clinical effectiveness of liquid aluminum-magnesium hydroxide gels have been reported by others (2–4) for the treatment of peptic ulcer, hyperacidity, heart burn, spasticity, and gastritis. Properly formulated they are very prompt and prolonged in antacid activity, maintain the gastric pH between 3 and 5, and do not exhibit significant diminution of antacid action on aging.

This investigation had as its purpose the preparation of aluminum-magnesium hydroxide gels in dry form which approaches the antacid activity of the clinically proved fluid gels.

Received April 28, 1961, from the Research Laboratory, Reheis Co., Inc., Berkeley Heights, N. J.
Accepted for publication September 6, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

EXPERIMENTAL

Preparation of Dried Gels.—Three series of aluminum-magnesium hydroxides were prepared. In each series the amount of aluminum to magnesium was varied in the atomic ratios of 2:1, 1:1, and 0.5:1.

The first series (AMH) consisted solely of aluminum and magnesium hydroxide. The second series (AMHG) was formulated to contain approximately 20% of glycine in the dried gel. The third series (AMHS) was prepared to contain 20% of sorbitol.

The method of preparation of all nine dried gels was similar. Highly reactive aluminum hydroxide containing some carbonate and pure gelatinous magnesium hydroxide were separately precipitated, the combined slurries were filtered and washed free of soluble salts, the mixed hydrogels were subjected to highly intensive shear at room temperature and reduced to finely divided powder form by either spray drying or low temperature air drying together with fine pulverizing. Glycine crystals or sorbitol solution was added to the mixed hydrogels before milling.

Physical Properties.—All the dried gels are soft, white, smooth, fine, tasteless, and odorless powders which react readily with gastric strength acid-containing pepsin. The AMH dried gels are somewhat less dense than those containing sorbitol or glycine. The pH of aqueous suspensions of the AMHG series is about 9.0—the others are about 9.6. The carbonate (as CO_2) content ranges from about 5.0 to 12% depending mainly on the method of drying. Dried gels containing glycine or sorbitol are less

varies even more (6.2%), even with the exclusion of the anomalous capsule No. 4 of low fill weight. Capsule No. 4 may either have a reasonable probability of occurrence or may be a maverick, possibly due to either improper weighing or being the first or last capsule prepared from the lot. Good control practice would demand repeat assays to clarify this.

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TABLE I.—COMPOSITION OF ALUMINUM-MAGNESIUM HYDROXIDE DRIED GELS

Type Designation	Atomic Ratio, Al:Mg	Approximate Analysis Al_2O_3, %	Approximate Analysis MgO, %	Relative Amount per Gm. Aluminum[a] Hydroxide U.S.P., mg.	Relative Amount per Gm. Magnesium Hydroxide N.F., mg.	Relative Amount per Gm. Glycine N.F., mg.	Relative Amount per Gm. Sorbitol, mg.	Ratio Aluminum Hydroxide to Magnesium Hydroxide	Acid[b] Consuming Capacity
F-AMH-21	2:1	39.4	15.8	773	227	...	...	3.4:1	305
F-AMH-11	1:1	32.1	25.6	629	371	...	...	1.7:1	310
F-AMH-12	0.5:1	23.4	37.4	459	541	...	...	0.85:1	314
F-AMHG-21	2:1	31.5	12.6	618	182	200	...	3.4:1	250
F-AMHG-11	1:1	25.7	20.4	504	296	200	...	1.7:1	254
F-AMHG-12	0.5:1	18.7	30.0	367	433	200	...	0.85:1	260
F-AMHS-21	2:1	31.5	12.6	618	182	...	200	3.4:1	250
F-AMHS-11	1:1	25.7	20.4	504	296	...	200	1.7:1	254
F-AMHS-12	0.5:1	18.7	30.0	367	433	...	200	0.85:1	200

[a] Calculated as 51% aluminum oxide. [b] Milliliters 0.1 *N* hydrochloric acid per Gm.

chalky. Spray dried samples have spherical shaped particles and are more free flowing.

Table I shows the composition of the nine aluminum-magnesium hydroxide dried gels as well as typical acid-consuming capacity values.

Infrared Studies.—Rock salt infrared spectra (2 to 15 μ) were run on samples of AMH-21, AMHG-21, AMHS-21, aluminum hydroxide dried gel U.S.P., magnesium hydroxide N.F., glycine N.F., and crystalline D-sorbitol. Nujol and hexachlorobutadiene (HCBD) mulls were made and samples run at capillary thicknesses *vs.* air.

Comparison of the various spectra shows that both AMH-21 and AMHS-21 are simple mixtures. The spectra of AMHG-21 indicates that it is predominantly a simple mixture of the three components, although there is strong evidence of a new glycine derivative—the percentage being fairly small.

Dry Blends.—In order to make a comparison between the antacid activity of the nine dried gels shown in Table I with usual dry blends, it was necessary to prepare nine such blends using aluminum hydroxide dried gel U.S.P., magnesium hydroxide N.F., glycine N.F., and crystalline sorbitol. The blends were made with finely powdered components.

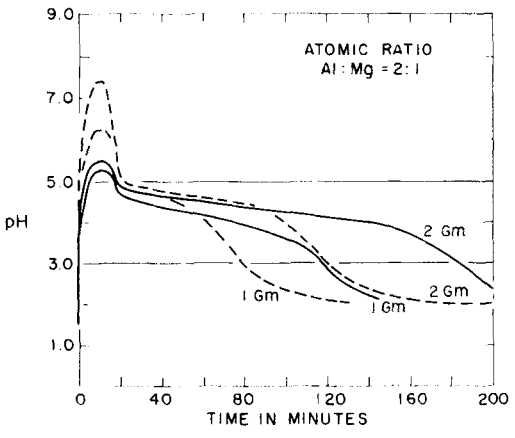


Fig. 1.—Antacid activity of aluminum-magnesium hydroxide dried gel Type F-AMH-21 (—) compared with dry blend of $Al(OH)_3$ U. S. P.-$Mg(OH)_2$ N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

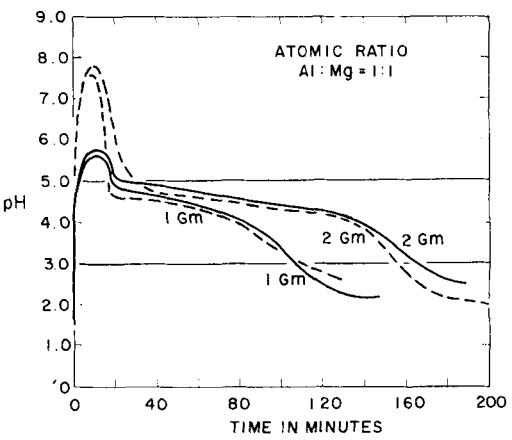


Fig. 2.—Antacid activity of aluminum-magnesium hydroxide dried gel Type F-AMH-11(—) compared with dry blend of $Al(OH)_3$ U. S. P.-$Mg(OH)_2$ N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

Antacid Activity.—The principal method used to evaluate the antacid activity of the nine aluminum-magnesium hydroxide dried gels, as well as the corresponding dry blends, was the dynamic, stringent procedure of Holbert, Noble, and Grote modified, which was described in considerable detail in the first paper in this series of *in vitro* studies (5). Samples were evaluated on the basis of 1.0 and 2.0-Gm. dose levels. The results for the various dried gels are plotted in Figs. 1-9.

Effect of Age.—To determine the effect of time on samples of aluminum-magnesium hydroxide dried gels stored in closed glass containers at ambient temperatures, the antacid activity of 1.0-Gm. samples was determined by the Holbert, Noble, and Grote method after 5 months' storage, and compared with values obtained on freshly prepared samples. The criteria used for aging is the total time that a 1.0-Gm. sample maintains the pH above 3.0 when tested by the modified Holbert, Noble, and Grote procedure. The results are shown in Table II.

Comparison with Dry Blends.—The antacid activity of 5-month-old samples of all nine AMH dried gels are compared with corresponding dry blends in Table II also.

Comparison with Liquid Gels.—For comparison

TABLE II.—EFFECT OF TIME ON ANTACID ACTIVITY[a] OF AMH DRIED GELS AND COMPARISON WITH DRY BLENDS

Type Designation	A Age of Sample, months 0 min.	B 5 min.	C Loss in Activity After 5 months, %	D Antacid Activity Corresponding Dry Blend, min.	Increase in Activity of Col. B over Col. D, %
F-AMH-21	142	113	20	68	66
F-AMH-11	142	102	28	97	3
F-AMH-12	135	127	6	128	same
F-AMHG-21	147	140	5	55	155
F-AMHG-11	142	133	6	88	51
F-AMHG-12	140	135	4	107	26
F-AMHS-21	145	134	8	47	185
F-AMHS-11	142	137	4	83	66
F-AMHS-12	150	143	5	105	36

[a] Procedure of Holbert, Noble, and Grote modified (time above pH 3.0 for 1.0-Gm. sample).

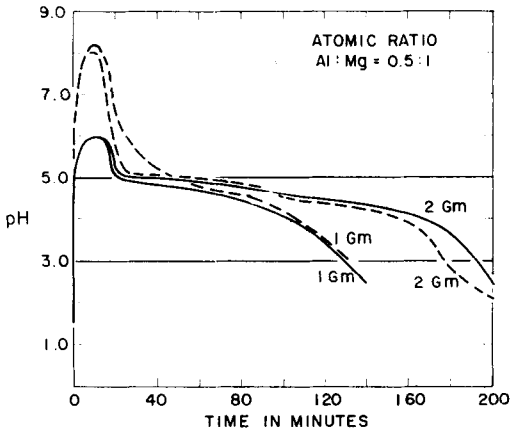


Fig. 3.—Antacid activity of aluminum-magnesium hydroxide dried gel Type F-AMH-12 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

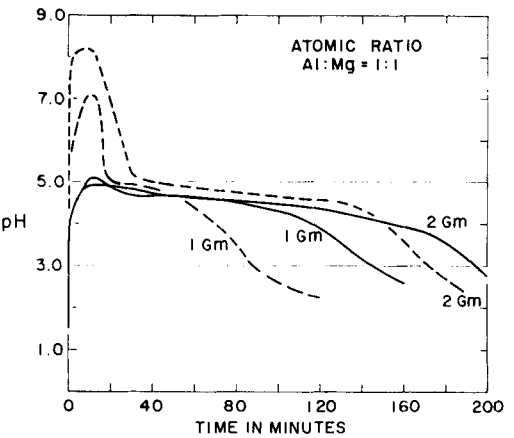


Fig. 5.—Antacid activity of aluminum-magnesium hydroxide-glycine dried gel Type F-AMHG-11 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–glycine N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

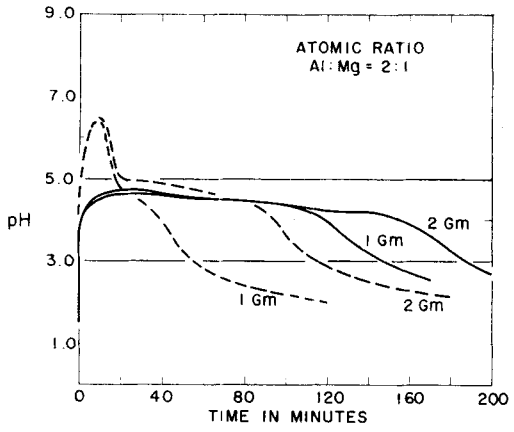


Fig. 4.—Antacid activity of aluminum-magnesium hydroxide-glycine dried gel Type F-AMHG-21 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–glycine N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

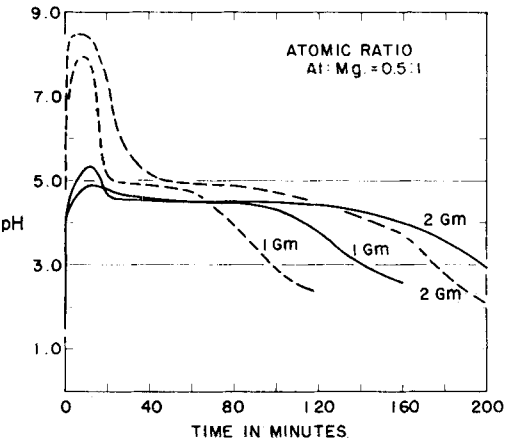


Fig. 6.—Antacid activity of aluminum-magnesium hydroxide-glycine dried gel Type F-AMHG-12 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–glycine N. F. (- - -). Procedure of Holbert, Noble, and Grote modified.

purposes the antacid activity data by the modified Holbert, Noble, and Grote method is plotted in Fig. 10 for 5, 10, and 15-ml. samples of a very reactive liquid aluminum-magnesium hydroxide gel having an Al:Mg atomic ratio of 1:1.

RESULTS

From consideration of the antacid activity data for the various aluminum-magnesium hydroxide dried gels and dry blends which are plotted in Figs.

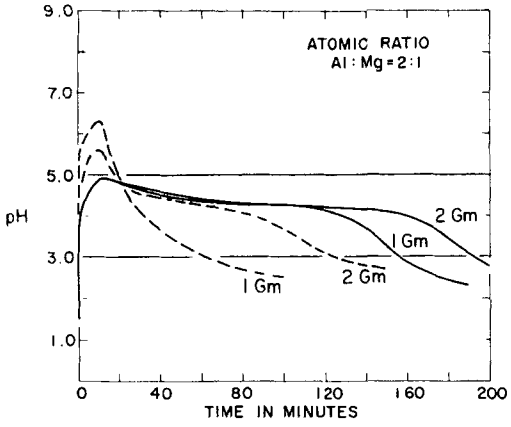


Fig. 7.—Antacid activity of aluminum-magnesium hydroxide-sorbitol dried gel Type F-AMHS-21 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–sorbitol (- - -). Procedure of Holbert, Noble, and Grote modified.

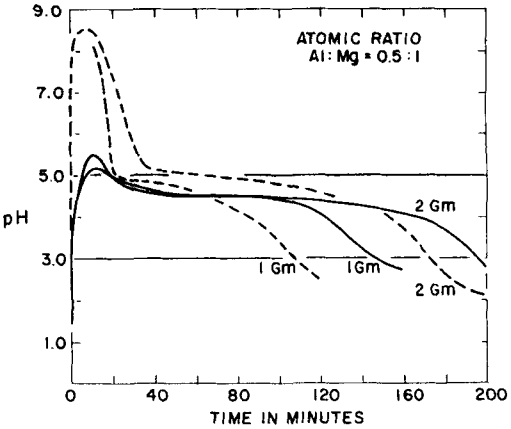


Fig. 9.—Antacid activity of aluminum-magnesium hydroxide-sorbitol dried gel Type F-AMHS-12 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–sorbitol (- - -). Procedure of Holbert, Noble, and Grote modified.

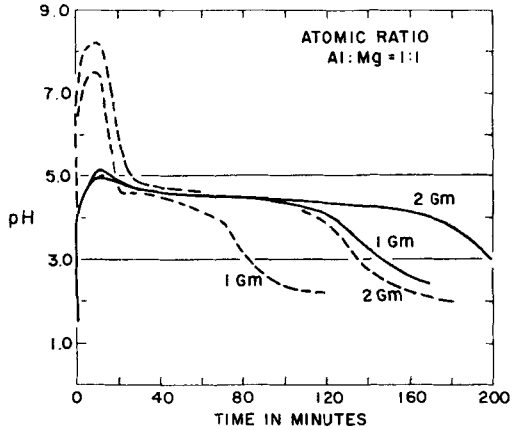


Fig. 8.—Antacid activity of aluminum-magnesium hydroxide-sorbitol dried gel Type F-AMHS-11 (—) compared with dry blend of $Al(OH)_3$ U. S. P.–$Mg(OH)_2$ N. F.–sorbitol (- - -). Procedure of Holbert, Noble, and Grote modified.

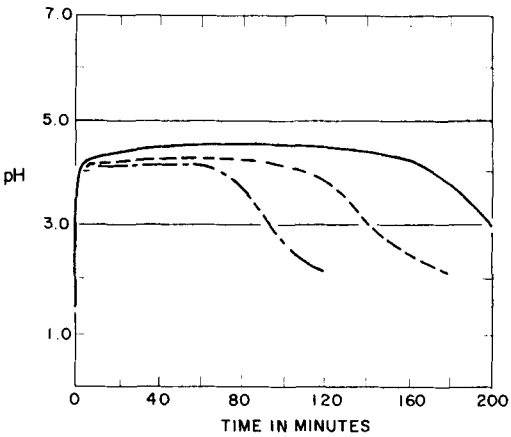


Fig. 10—Antacid activity of liquid aluminum magnesium hydroxide gel. Procedure of Holbert, Noble, and Grote modified. —, 15 ml.; - - -, 10 ml.; — — —., 5 ml.

1–9 and compared in Table II, one may draw several conclusions as follows: (*a*) Aluminum-magnesium hydroxide dried gels prepared by the new process described are much more prolonged in antacid activity in the optimum pH range of 3 to 5 than corresponding dry blends containing the same basic components in the same proportions. They also do not cause such a high undesirable initial rise in pH. (*b*) Unstabilized aluminum-magnesium hydroxide dried gels in the Al to Mg atomic ratio range of 2:1 and 1:1 show a fairly substantial diminution in antacid activity after 5-months' aging. This is a characteristic also of aluminum hydroxide dried gel U.S.P. Acid consuming capacity tests do not show this aging effect. (*c*) Glycine and sorbitol are both excellent stabilizers of the complex aluminum-magnesium hydroxide gel micellar structures during the desiccation process. On freshly prepared samples AMHG and AMHS dried gels have about the same antacid activity as an equal weight of unaged, unstabilized AMH dried gel, although they contain only 80% as much aluminum and magnesium hydroxide. When 5-month-old samples of all dried gels are compared on an active ingredient basis, the AMHG and AMHS dried gels in the Al:Mg ranges of 2:1 and 1:1 are 55–62% more active and 29–33% when the Al:Mg ratio is 0.5 to 1.

DISCUSSION

Aluminum-magnesium hydroxide dried gels AMHG and AMHS approach the theoretical requirements for an ideal antacid in dry form because of the following factors: (*a*) they are very rapid in raising the pH of gastric juice to the optimum pH range of 3 to 5, (*b*) they have extremely long periods of times of reaction in this optimum range, (*c*) the high initial rise in pH characteristics of similar materials dry blended is avoided, (*d*) they are nonsystemic and hence cannot upset the acid-base

balance of the blood, (*e*) they exhibit very little diminution of antacid activity on aging, (*f*) they are not adversely affected by pepsin in antacid action, (*g*) they are nonirritating to the gastrointestinal tract, (*h*) they have a desirable mild astringent effect, (*i*) they have minimal constipating or laxative effects, (*j*) they are palatable with very little chalkiness, (*k*) they rehydrate and swell in water to provide a gelatinous, positively charged protective coating for inflamed membranes, and (*l*) they may be produced at moderate cost because of the uncomplicated chemical nature of the compounds employed.

SUMMARY

The preparation of a group of new highly reactive and stable aluminum-magnesium hydroxide gels in powder form has been described. The determination of their antacid activity by a very stringent *in vitro* procedure indicates that they compare favorably with clinically effective liquid aluminum-magnesium hydroxide gels. The dried gels are effective over a wide range of proportions of the two antacid components. The new dried gels which are predominantly simple mixtures and not new compounds approach the ideal for an antacid in dry form.

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Preparation and Properties of New Gastric Antacids VI

Low Sodium Aluminum Hydroxide Gel and Dried Gels

By STEWART M. BEEKMAN

The preparation of a new low sodium aluminum hydroxide concentrated gel is described, which when diluted to U.S.P. concentration contains about 0.6 mg. of sodium per 30 ml. The preparation of six new aluminum hydroxide dried gels containing 0.19 to 0.50 mg. of sodium per Gm. is also described. They include an aluminum hydroxide dried gel U.S.P. as well as four combinations with magnesium hydroxide and one with magnesium carbonate. The antacid activity of the various preparations was determined by a very stringent *in vitro* procedure. The activity of the combination dried gels compares favorably with the liquid gel. They are rapid and very prolonged in the optimum pH range of 3 to 5.

RECENT reports (1, 2) in the medical literature have stressed the need for low sodium antacids in liquid and tablet form for the treatment of patients who may have peptic ulcers or bleeding esophogeal varices concomitant with liver or heart disease that may require sodium restriction. Rimer and Frankland (1) who have given this problem detailed study comment that "in prescribing antacids, one may unwittingly violate the first principle of therapeutics and do unnecessary harm to the patients." Bleifer and associates (2) report that "it is obvious that unrestricted use of these materials (antacids) can jeopardize investigative sodium studies as well as therapeutic salt restricted programs."

Rimer and Frankland reported on the analyses of 18 of the most widely used aluminum hydroxide preparations in liquid and tablet form and found that the liquids contain 11.9 to 269.7 mg. of sodium per 30 ml. and from 5.1 to 192 mg. of sodium per 8 tablets. Bleifer, *et al.*, found that four of the popular liquid aluminum hydroxide gels contained 29–39 mg. of sodium per 30-ml. dose. Since the daily dose for patients with acute ulcers may be 500 ml. or more, the amount of sodium ingested from this source alone may be

Received April 28, 1961, from the Research Laboratory, Reheis Co., Inc., Berkeley Heights, N. J.
Accepted for publication September 6, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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500–2500 mg. per day. Many physicians try to restrict the sodium intake from all sources to 210 mg. per day.

The purpose of this paper is to report the results of an investigation which had as its goal the preparation of clinically effective aluminum hydroxide gel and various dried gels with low sodium contents.

EXPERIMENTAL

Aluminum Hydroxide Gel.—A stable, highly reactive aluminum hydroxide was precipitated from the system K_2CO_3-$KHCO_3$-$AlCl_3$ using sodium free aluminum chloride and deionized water. The gel was filtered, washed free of soluble salts, and subjected to high shear at room temperature under aseptic conditions. For the preparation of aluminum hydrogels having uniform physical and chemical properties it is necessary to control many variables carefully including concentration and precise metering of reactants, temperature, degree and type of agitation, reaction rate, method of introducing reactants, pH change, and aging. The resulting snow white, homogeneous hydrogel contains about 9% aluminum hydroxide as aluminum oxide, and when diluted to 4.0% aluminum oxide forms a flowable, slow settling aluminum hydroxide U.S.P. of proper viscosity characteristics. It is designated as type F-1000 LS. Table I shows the analysis of a typical sample diluted to U.S.P. concentration.

Aluminum Hydroxide Dried Gel.—Aluminum hydroxide gel similar to that described above was carefully dried by low temperature circulating air and pulverized to a finely divided form. The resulting soft, white, bulky powder meets the requirements for a fairly reactive aluminum hydroxide dried gel U.S.P. It is also designated as type F-1000 LS. The analysis of a typical sample is seen in Table I.

Aluminum-Magnesium Hydroxide Dried Gels.—Low sodium magnesium hydroxide was precipitated, mixed with low sodium aluminum hydroxide gel, combined with either glycine or sorbitol, subjected to intensive mixing, and reduced to dry powder form. Four different dried gels were prepared. Two were stabilized with glycine and two with sorbitol. The preparations were made with Al:Mg atomic ratios of 2:1 and 1:1. In Table I the analyses of typical samples are shown.

Aluminum Hydroxide-Magnesium Carbonate Dried Gel.—Magnesium carbonate gel was prepared by the low temperature precipitation in the system K_2CO_3-$MgCl_2$, intimately combined with low sodium aluminum hydroxide gel, and the resulting mixture spray dried to finely divided form. The analysis of a typical sample of this dried gel which is designated as Type F-MA11-LS may be seen in Table I.

Sodium Content.—The sodium content of the various samples was determined by means of a Beckman model B spectrophotometer with flame attachment. The results are also shown in Table I.

Antacid Activity.—The acid consuming capacity (ACC) values for all samples are shown in Table I. Since all samples were completely soluble in 0.1 *N* hydrochloric acid at 37.5°, the ACC values are approximately equivalent to the amount of aluminum and/or magnesium present in the various products.

Reheis and Mutch reaction velocity values (3) were determined on all samples. They were seen to be rapidly reactive. The least reactive was the aluminum hydroxide dried gel U.S.P.

The principle method of measuring the antacid activity was the modified Holbert, Noble, and Grote procedure previously described, using an automated apparatus developed for the purpose. Figure 1 shows the results on 5, 10, and 15-ml. samples of an aluminum hydroxide gel U.S.P., Type F-1000 LS. In Fig.2 the antacid activity of new and 4-month-old samples of an aluminum hydroxide dried gel U.S.P., Type F-1000 LS is plotted on the basis of 1.0 Gm. Figure 3 shows the results obtained on 1.0-Gm. samples of the two aluminum-magnesium hydroxide glycine dried gels. The data for the two aluminum-magnesium hydroxide sorbitol dried gels is seen in

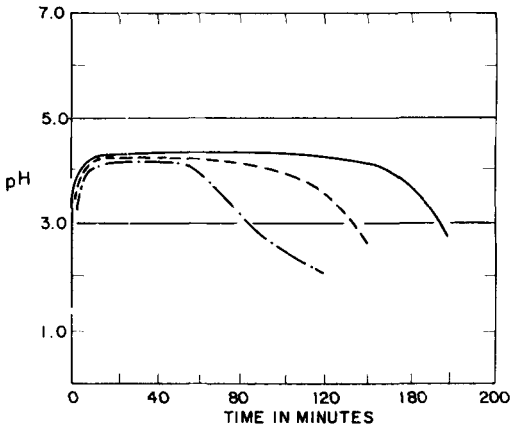


Fig. 1.—Antacid activity of low sodium aluminum hydroxide gel U.S.P, type F-1000LS. ——, 15 ml.; - - -, 10 ml.; -·-·, 5 ml., procedure of Holbert, Noble, and Grote modified.

TABLE I.—ANALYSIS OF TYPICAL SAMPLES OF LOW SODIUM ALUMINUM HYDROXIDE GELS AND DRIED GELS

Type Designation	Al_2O_3, %	MgO, %	Glycine, %	Sorbitol, %	CO_2, %	Na, %	Acid[c] Consuming Capacity
F-1000-LS[a]	4.0	...	...	..	1.7	0.0019	22.6
F-1000-LS[b]	52.1	...	...	..	10.5	0.019	302
F-AMHG-21LS	33.4	12.6	19.1	..	7.1	0.019	264
F-AMHG-11LS	28.2	21.6	19.5	..	7.1	0.029	279
F-AMHS-21LS	32.9	13.7	...	20	6.8	0.029	253
F-AMHS-11LS	27.0	22.8	...	20	6.7	0.029	271
F-MA11-LS	38.1	9.5	...	..	22.1	0.05	272

[a] Aluminum hydroxide gel U.S.P. [b] Aluminum hydroxide dried gel U.S.P. [c] Milliliters 0.1 *N* hydrochloric acid per Gm.

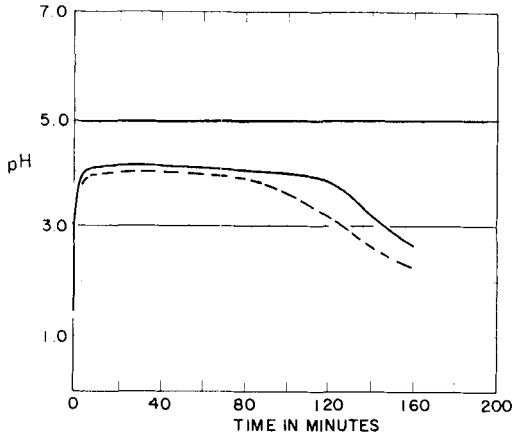


Fig. 2.—Antacid activity of low sodium aluminum hydroxide dried gel U.S.P., Type F-1000LS, basis 1.0 Gm. Age of sample: ——, 4 days; - - -, 4 months. Procedure of Holbert, Noble, and Grote modified.

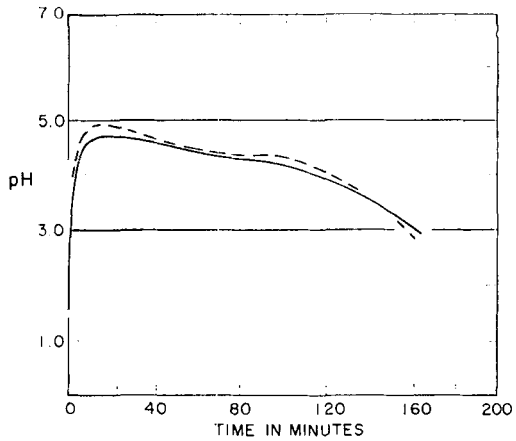


Fig. 3.—Antacid activity of two low sodium aluminum-magnesium hydroxide glycine dried gels, basis 1.0 Gm. ——, F-AMHG-11LS; - - - F-AMHG-21LS. Procedure of Holbert, Noble, and Grote modified.

Fig. 4. Lastly, the antacid activity of the F-MA11-LS dried gel is seen in Fig. 5.

In Table II the antacid characteristics of the various low sodium dried gels are compared on the basis of 1.0-Gm. dose levels. The data is derived from the time-pH curves shown in Figs. 2–5.

RESULTS

Aluminum Hydroxide Gel.—The data presented show that a concentrated aluminum hydroxide gel may be prepared which when diluted to U.S.P. concentration of 4.0% aluminum oxide contains about 0.6 mg. of sodium per 30 ml. It can be seen that a massive 500-ml. daily dose would add only 10 mg. of sodium to the dietary intake, and hence could not be expected to cause edema or ascites in heart or liver disease patients. The gel is prepared from nonsodium reactants and should add less than two cents to the manufacturing cost of a standard 12-oz. bottle of liquid antacid.

Aluminum Hydroxide Dried Gels.—The sodium content of the various dried gels is seen in Table I to vary from 190 to 500 p.p.m. which would add, e.g., only 1 to 2.5 mg. of sodium for 8–10-gr. tablets. As might be expected from data presented in previous papers (3–5) in this series of *in vitro* studies, the aluminum hydroxide dried gels containing either magnesium carbonate or magnesium hydroxide stabilized with glycine or sorbitol exhibit antacid activities which are similar to reactive liquid aluminum hydroxide gel. The aluminum hydroxide dried gel U.S.P. type F-1000 LS prepared as above was much more reactive than most aluminum hydroxide dried gels U.S.P. that we have evaluated by our automated *in vitro* technique. However, after 4 months' aging it had declined in initial speed of reaction some 25%, and 14% in duration of antacid action above pH 3.0.

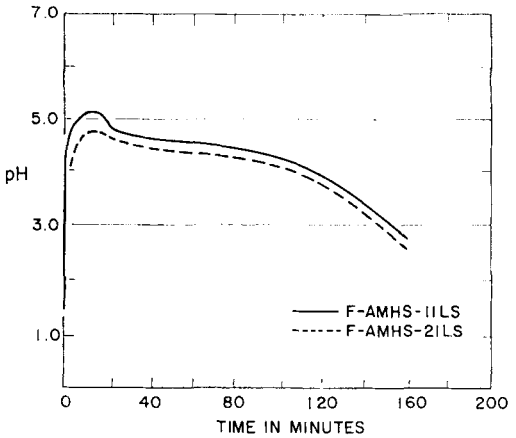


Fig. 4.—Antacid activity of two low sodium aluminum-magnesium hydroxide sorbitol dried gels, basis 1.0 Gm. ——, F-AMHS-11LS; - - - F-AMHS-21LS. Procedure of Holbert, Noble, and Grote modified.

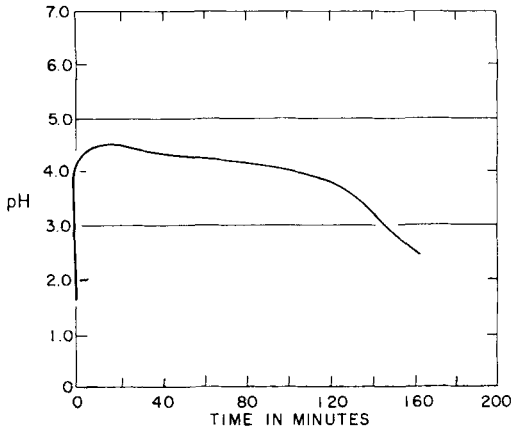


Fig. 5.—Antacid activity of low sodium aluminum hydroxide-magnesium carbonate dried gel type F-MA11-LS, basis 1.0 Gm. Procedure of Holbert, Noble, and Grote modified.

TABLE II.—COMPARISON OF ANTACID ACTIVITY[a] OF VARIOUS LOW SODIUM ALUMINUM HYDROXIDE DRIED GELS

Sample	Time to Reach pH 3.0, sec.	Max. pH	Total Time above pH 3.0, min.
F-1000-LS age, 4 days	180	4.1	145
F-1000-LS age, 4 months	450	4.0	125
F-AMHG-21 LS	45	4.7	160
F-AMHS-21 LS	45	4.7	140
F-AMHG-11 LS	40	4.9	153
F-AMHS-11 LS	38	5.2	150
F-MA11- LS	10	4.6	145

[a] Data from time-pH curves for 1.0-Gm. samples. Procedure of Holbert, Noble, and Grote modified.

DISCUSSION

Nature of Aluminum Hydrogels.—The low sodium antacids prepared in this study compare favorably in all important properties with similar preparations manufactured with sodium salts. The nature of aluminum hydrogels are such that they readily adsorb foreign cations and anions from the systems in which they are made. Many of these ions are strongly bound and are not readily desorbed by washing copiously with pure water. Nor is it entirely desirable to do so, since their excellent antacid action and stability depends in large measure on the nature and amount of these adsorbed impurities.

Economics and Demand for Low Sodium Gels.—The substitution of potassium salts for sodium salts requires some minor changes in their manufacturing process particularly in the precipitation step and adds to the raw material cost because they are heavier in molecular weight and higher in price than sodium salts. Since other costs such as processing, container, shipping, etc., are similar for both systems, the price of low sodium products to the pharmaceutical manufacturer probably should not exceed 25% of the prices for present sodium-containing aluminum hydroxide gels.

One important variable is the potential volume for such low sodium products. As Bleifer, *et al.* (2), point out "the small concentrations of sodium present are in no way contraindicated in the overwhelming majority of cases in which antacid therapy is needed."

It may be uneconomical for manufacturers to make and pharmacists to stock both regular and low sodium grades. It is possible that a demand will be made in the future for sodium content statements on labels of all antacids destined for therapeutic use.

SUMMARY

1. A new concentrated aluminum hydroxide gel has been prepared which contains about 19 p.p.m. of sodium when diluted to U.S.P. strength of 4.0% aluminum oxide. The physical properties of the gel such as viscosity, settling characteristics, taste, and color are excellent. The antacid activity characteristics were determined by a very stringent *in vitro* procedure and seem to compare well with usual high sodium aluminum hydroxide liquid preparations similarly tested.

2. The preparation of six new aluminum hydroxide dried gels containing 190 to 500 p.p.m. of sodium is described. Four co-dried mixtures of aluminum hydroxide with magnesium hydroxide and glycine or sorbitol as well as a co-dried blend with magnesium carbonate are seen to have antacid characteristics similar to liquid aluminum hydroxide gels. An aluminum hydroxide dried gel U.S.P. with fairly good antacid properties is also described.

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Aggregation in Oil-in-Water Emulsions

Effects of Dioctyl Sodium Sulfosuccinate Concentration

By W. I. HIGUCHI, RYUZO OKADA, and A. P. LEMBERGER

A procedure was developed which is suitable for the study of aggregation in oil-in-water emulsions. It involves the preparation of a relatively monodispersed emulsion (droplet sizes around 1 μ diameter) by the electrical dispersion technique and the determination of the distribution of sizes of the aggregates with the Coulter counter. The method was applied to the study of aggregation in hexadecane-in-water emulsions containing dioctyl sodium sulfosuccinate[1] (AOT) at varying concentrations. It was found that at low AOT concentrations ($\lesssim$0.1%) the emulsions were completely deaggregated. As the AOT concentration increased beyond this value, aggregation set in and progressively increased over the entire range of AOT concentration studied (up to 1.5%). In this system, aggregation was found to be completely reversible, the equilibrium being reached in all cases in a few days.

THE AGGREGATION of droplets in an emulsion or of particles in a suspension may alter the dynamic behavior of the system in at least two basic ways. First, the rate of creaming or sedimentation in dilute systems will be very much greater when aggregation is present (1). Since gravitational effects are much greater in creamed or sedimented systems, the rates of coalescence in emulsions and the caking rates in suspensions will be expected to take place more rapidly. Secondly, aggregation may affect the rheology (2) of the system. In general one would expect either thixotropic or pseudoplastic behavior in aggregating systems. The greatest amount of aggregation should be present at zero shear, and as the shear rate increases, deaggregation will increase as the forces of shear tend to pull apart the aggregates. Whether the system will be thixotropic or pseudoplastic depends upon how rapidly the aggregation-deaggregation equilibrium is established.

Since both the physical stability and flow properties of emulsions and suspensions are of great interest to the development pharmacist, a basic quantitative study of aggregation is essential. To the authors' knowledge, all past studies (1, 3, 4) of this problem for particles and droplets in the micron range or greater have not been of direct nature. Generally, creaming or sedimentation rates were followed and the aggregation behavior deduced in a qualitative or semiquantitative fashion. Because the situation in detail is very complex, to understand truly the important factors involved in aggregation, the phenomenon itself should be studied as directly as possible.

The purpose of this communication is twofold. A main purpose is to present a procedure which is well suited to quantitative studies of emulsion aggregation. It involves the preparation of a relatively narrow size range oil-in-water emulsion and the rapid counting and sizing of the droplets as a function of time. Secondly, the method is applied to the investigation of the the effects of the surfactant (dioctyl sodium sulfosuccinate) concentration on aggregation and deaggregation in hexadecane-in-water emulsions.

THEORY AND PAST EXPERIMENTS

Aggregation in emulsion systems is the formation of agglomerates without coalescence, i.e., without the actual rupture of the interfacial film and the subsequent formation of larger droplets. When contact of phases is prevented by a sufficiently high barrier, which may be either electrical or steric in nature, coalescence can be prevented. However, aggregation may take place in these systems if the attractive droplet-droplet forces[2] predominate at long range.

This long range attraction may be looked upon as arising from either or both of two distinct sources. First of all, two droplets will attract each other appreciably (attractive energies $\gtrsim kT$, where k = Boltzmann's constant and T = absolute temperature) even at distances large compared to molecular dimensions. These forces are the London dispersion forces accentuated as a result of the integration of the molecule-molecule interactions over the volumes of the two droplets. Studies both theoretical (5, 6) and experimental (3) clearly expose the existence of these macroscopic forces. The second contribution to droplet-droplet attraction might arise if, in the region between the two particles, the interfacial film of one droplet attracts the film of the other. This would be expected to be most important when the

Received April 28, 1961, from the University of Wisconsin, School of Pharmacy, Madison.
Accepted for publication August 4, 1961.
Work supported in part by a grant from Parke, Davis & Co., by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by a grant from the National Institutes of Health A2649(cl).
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

[1] Marketed as Aerosol OT by American Cyanamid Co.

[2] These may include interactions involving adsorbed films when surfactants are present or if surface solvation is important.

absorbed surfactant films on oil droplets in water expose relatively hydrophobic and/or nonionizing functional groups toward the water phase. "Soluble" surfactants which, when adsorbed onto oil droplets, are able to expose an abundance of hydrophilic groups to the water should, in general, not contribute to droplet-droplet attraction, and therefore aggregation. On the contrary, by means of their charges or/and hydrophilic bulk, they should contribute to the prevention of close approach of the droplets.

If, then, the repulsive forces (electrical or steric) are not as long ranged as the above attractive forces, the droplets may find themselves energetically more comfortable in the aggregated states.

Cockbain (1) has interpreted his creaming rate studies of benzene- and paraffin-in-water emulsions in the following way: conventional low molecular weight ionic stabilizers, both cationics and anionics, prevent both coalescence and aggregation at low stabilizer concentrations ($\lesssim$0.1% or so), but at higher concentrations (~0.1 to 1% or so) aggregation occurs in all cases. At much higher concentrations the droplets deaggregate in some cases. Because without exception aggregation was found with all surfactants studied, the practical importance of Cockbain's observations are clear.

EXPERIMENTAL

General Considerations.—The following novel measures were taken to permit a direct study of aggregation and deaggregation. To eliminate as much as possible the effects of the distribution of sizes, narrow size range emulsions were prepared by the high voltage electrical dispersion method described by Nawab and Mason (7). To follow aggregation, the Coulter counter[3] (8) was employed to determine both droplet concentration and size distribution changes as a function of time.

Preparation of Emulsions.—Hexadecane was redistilled Eastman practical grade. The American Cyanamid "100% pure" Aerosol OT (AOT) was specially purified (8) by fractional precipitation from methanol and water-methanol solvent mixture. The water used was doubly distilled.

Hexadecane containing 2.0% by weight AOT was placed in a syringe, as shown in Fig. 1. A No. 25 needle, with the tip evenly cut off perpendicular to its axis, permitted a flow of about 20 drops per second with no applied voltage to the needle. The hexadecane solution was sprayed onto a 1% aqueous AOT solution with 6 kv. applied to the needle. The aqueous solution was grounded as shown and was agitated with a magnetic stirrer. The needle tip-to-solution surface distance was 2.5 cm. These conditions were selected to give the best appearing spray. The spraying was continued until about 40% by volume hexadecane had been emulsified. This was the stock emulsion used in the studies. It was kept in a polystyrene bottle at 30°C. in a rotating wheel type constant temperature bath which provided gentle agitation.

Calibration and Use of the Coulter Counter.—The 30-μ aperture was used throughout these studies. The threshhold scale of the instrument was calibrated with polystyrene latices,[4] 1.17 and 1.83 μ diameters,[5] at all sensitivity and attenuator settings used in our studies except for those corresponding to low sensitivities. These latter settings were calibrated by the data themselves by determining the scale factors which gave the same count *vs.* threshhold values. It was assumed that the pulse height was proportional to the volume of the particle passing through the aperture. This is the usual assumption which may be justified theoretically in an approximate manner. Also, employing blood cells as a third calibration point this assumption has been approximately verified by us in at least one instance.

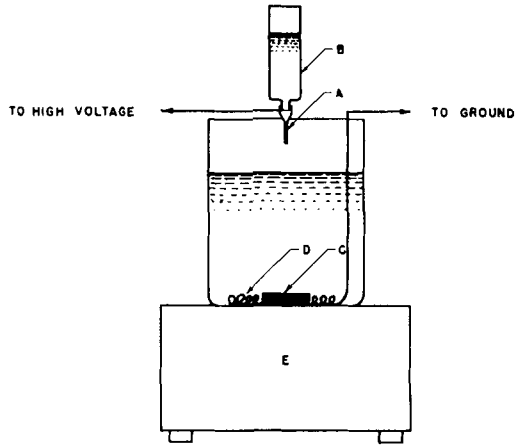


Fig. 1.—Apparatus for the preparation of emulsions by electrical dispersion. A, hypodermic needle; B, syringe; C, stirring bar; D, grounding coil; E, stirrer.

In Fig. 2, results of a typical calibration run of a mixture of 1.83 and 1.17 μ latices in 0.90% saline solution are shown. The curve shown is the derivative[6] of the cumulative count curve (counts *vs.* threshhold curve), which is the one directly obtained with the instrument. Since the standard deviation of the latex sizes was known to be considerably less than the peak widths and since these particles did not appear to be agglomerated to any extent, the peak widths are believed to be a measure of the resolving power of the instrument. Thus, it is apparent that the resolving power of the instrument was about 0.2 μ in this range.

Procedure for Deaggregation Studies.—The stock emulsion (see above) was diluted to 1.0 and 2.0% by volume hexadecane with water containing varying amounts of AOT so that the final emulsions contained AOT ranging from about 0.05 to 1.5% by weight. About 10 ml. of these emulsions was pipetted into leak-tight vials of about 15-ml. capacity. The vials were fixed to wheels rotating at 30 r.p.m. in a constant temperature bath maintained at 30°C.

[3] Coulter Industrial Sales, Elmhurst, Ill.

[4] Kindly supplied by J. W. Vanderhoff, Dow Chemical Co., Midland, Mich.

[5] These are electron microscope values. It was assumed that the aqueous suspension sizes were the same.

[6] These and those for the emulsions were obtained by numerical difference methods. While the distribution "curves" should really be histograms for this reason, intervals taken on the *x*-axis were generally small enough so that the "smoothing" introduced little error for our purposes.

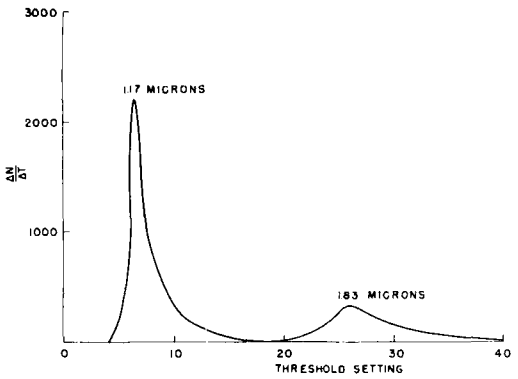


Fig. 2.—Differential count curve for a mixture of 1.17 and 1.83 μ diameter polystyrene spheres obtained with the Coulter counter. The ordinate is the derivative of the cumulative count curve with respect to the threshold setting.

Samples were removed from time to time and the size analyses with the Coulter counter carried out in the following way. The 1.0 and 2.0% hexadecane emulsions were diluted by about a factor of 1000 to provide a low enough concentration suitable for the Counter. To accomplish this without sacrifice of volumetric accuracy, the dilution was carried out in two steps, *viz.*, 1 to 10 with water followed by a 1 to 100 in 0.90% saline.

Procedure for Aggregation Studies.—Emulsions in which aggregation was completely absent (AOT concentrations $\lesssim 0.09\%$) were aggregated to varying degrees by addition of solid AOT. These were handled in the same manner as in the deaggregation experiments. Samples were taken over a period of about 10 days.

RESULTS

Figure 3 gives the number-diameter distribution curve for the completely deaggregated emulsion. Complete deaggregation was observed for the 1.0 and 2.0% emulsions when the AOT concentration was 0.09% or less. This curve shows the remarkable ability of the high voltage technique to prepare emulsions with a very high population of essentially a single size. It should be emphasized, however, and it is apparent from the curve, that the mass distribution curve will appear considerably different, being much broader. In all cases, at maximum deaggregation the 0–5 μ range accounted for only about 50% of the total hexadecane used in the preparation. Most of this discrepancy could be accounted for by extrapolation of the distribution curve to about 10 μ. Presumably, some of these large droplets were formed not in the spraying but from the portions of the hexadecane which dropped from the needle prior to and after the high voltage was applied and which were subsequently emulsified by the stirring.

Figures 4–7 give the distribution curves showing deaggregation as a function of time for the 1.0% emulsions at different AOT concentrations. After about 10 days, aggregation-deaggregation equilibrium was apparently reached in all cases. Distribution curves obtained at later times did not show further deaggregation. It is noteworthy that only after the stock emulsion had itself equilibrated for about 1 week did the results of the deaggregation experiments become reproducible. Apparently the redistribution kinetics of the AOT was very slow in this concentrated and highly aggregated system.

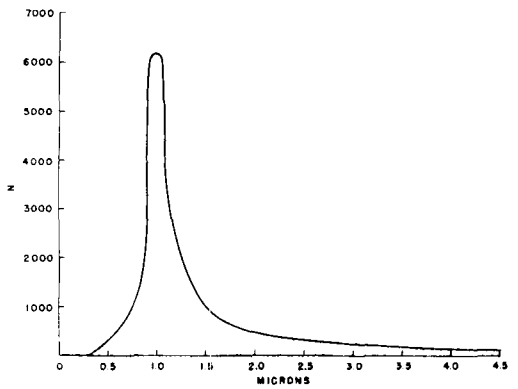


Fig. 3.—The droplet diameter distribution curve for the completely deaggregated hexadecane-in-water emulsion prepared by the electrical dispersion method.

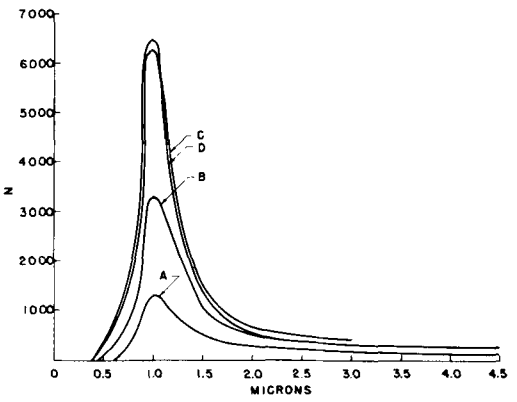


Fig. 4.—Deaggregation in 1.0% hexadecane emulsion containing 0.045% AOT when diluted from stock emulsion. A, initial; B, 3 days; C, 6 days; D, 9 to 12 days.

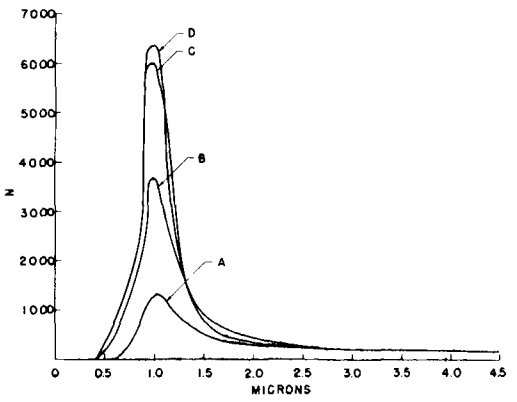


Fig. 5.—Deaggregation in a 1.0% hexadecane emulsion containing 0.09% AOT. A, initial; B, 3 days; C, 6 days; D, 9 to 12 days.

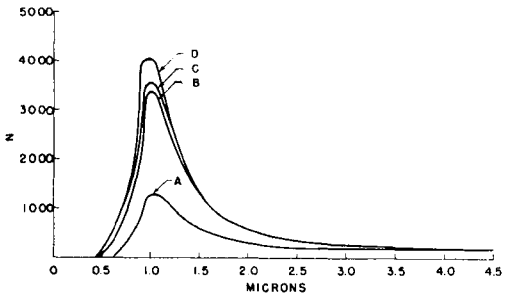


Fig. 6.—Deaggregation in a 1.0% hexadecane emulsion containing 0.50% AOT. A, initial; B, 3 days; C, 6 days; D, 9 to 12 days.

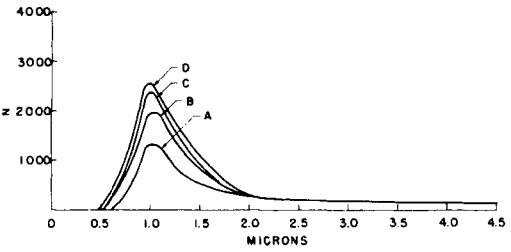


Fig. 7.—Deaggregation in a 1.0% hexadecane emulsion containing 1% AOT. A, initial; B, 3 days; C, 6 days; D, 9 to 12 days.

It can be seen from Figs. 4–7 that during the initial periods of deaggregation not all of the final mass of the completely deaggregated emulsion can be found in the 0–5 μ range. This is in contrast to the aggregation data for the 2.0% emulsions summarized in Figs. 8 to 11, in which cases the disappearance of the single particles in the 1 μ size region may be accounted for by the increases in concentration of particles in the 1.5 to 4 μ size range. The difference in behavior may be explained by the existence of very large aggregates in the stock emulsion. These would be expected to deaggregate much more slowly than the smaller aggregates.

That aggregation-deaggregation was completely reversible was demonstrated by the following experiment. A completely deaggregated 2.0% hexadecane

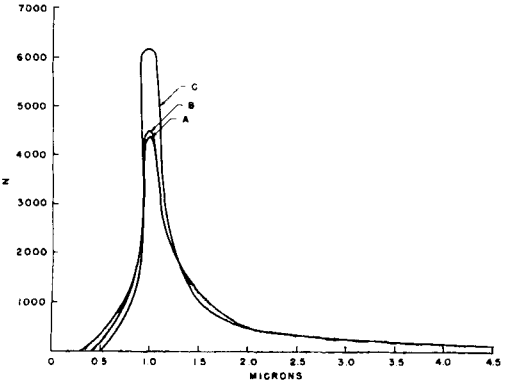


Fig. 8.—Aggregation in an initially completely deaggregated 2.0% hexadecane emulsion containing 0.18% AOT. C, initial; B, 2 days; A, 4 to 7 days.

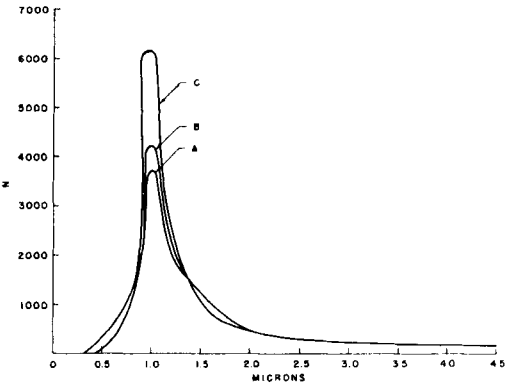


Fig. 9.—Aggregation in a 2.0% hexadecane emulsion containing 0.29% AOT. C, initial; B, 2 days; A, 4 to 7 days.

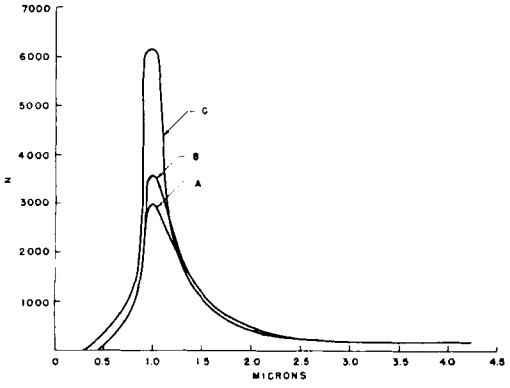


Fig. 10.—Aggregation in a 2.0% hexadecane emulsion containing 0.49% AOT. C, initial; B, 2 days; A, 4 to 7 days.

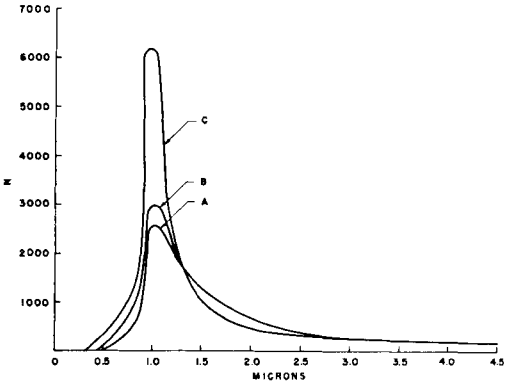


Fig. 11.—Aggregation in a 2.0% hexadecane emulsion containing 0.99% AOT. C, initial; B, 2 days; A, 4 to 7 days.

emulsion containing 0.09% AOT was partially aggregated by addition of enough AOT so that the AOT concentration was 0.18%. This emulsion was then diluted by a factor of two with water. The initial and final emulsions gave identical size distribution curves.

Earlier in these studies there was some concern as to whether the degree of aggregation in the 1.0 and 2.0% emulsions may have been altered by the dilution steps (see *Procedure*) themselves. Specifically, the questions were (*a*) did the first dilution step deaggregate the emulsions to any extent and (*b*) did the high salt concentration in the second step of dilution induce coalescence, further aggregation, or deaggregation? By varying the time of mixing from a few minutes to 30 minutes, no effect of the first dilution step on the results was observed. Also for periods up to 1 hour, no change in the counting was observed after the second dilution. Furthermore, the manner of introduction of sample and the degree of stirring during the introduction was unimportant for the second dilution. Thus, true pictures of the 1.0 and 2.0% emulsions were very likely obtained by the procedure employed.

DISCUSSION

Method.—The above results demonstrate that this procedure is suitable for quantitative aggregation studies. It should prove to be useful and practical in the evaluation of additives as to their ability to prevent aggregation and coalescence. The relative rapidity of the measurements permits the determination of the distribution of sizes within minutes. This is not only convenient but allows studies of relatively rapidly changing systems with apparently little disturbance to the systems themselves.

Mechanism of Aggregation in Hexadecane Emulsions Stabilized by AOT.—Cockbain (1) has proposed that aggregation in this system results possibly from polymolecular adsorption of the surfactant on the droplet surface. He suggests that after the formation of a monolayer, which exposes primarily hydrophilic groups to the aqueous phase, a second layer begins in which the molecules are oriented in the reverse direction, i.e., with the hydrophobic groups oriented toward the aqueous phase. Thus, interactions between these hydrophobic portions of the second layer of the droplets may account for aggregation.

While the above picture may be the correct one, the following alternative mechanism similar to that of Greiner and Vold (4) may be proposed which does not invoke the second and hydrophobic layer concept. At sufficiently low surfactant concentrations (<0.1% AOT), a monolayer or less of surfactant is adsorbed and aggregation is prevented by the repulsive long range interaction of the electrical double layers around the droplets. The double layer arises because the sulfonate ion is preferentially adsorbed and the sodium ions are distributed according to the Poisson-Boltzmann equation. If the counterion (Na^+) concentration is low enough, the thickness of the electrical double layer will be great and thus long range repulsion will prevail and cancel out the London attractive forces to within about kT for all interparticle distances. Thus aggregation is prevented at low AOT concentrations.

Now as the AOT concentration increases, the Na^+ concentration increases in the system. This increase in Na^+ concentration is approximately equal to the AOT concentration increase up to the critical micelle concentration (CMC), beyond which the increase in Na^+ will be slower because the dissociation of the Na^+ from the micelles will be limited. As the Na^+ concentration increases, the thickness of the double layer will decrease by at least, approximately, the inverse square root of the Na^+ concentration. When the thickness of this electrical layer is sufficiently reduced so that the range of the repulsive forces have become sufficiently reduced, the London attractive forces will predominate by more than kT at some interparticle distances. This is the secondary minimum phenomenon (3, 5, 6). Under these conditions aggregation may take place. The depth of this minimum and, hence, the forces of particle-particle attraction will increase with increasing Na^+ concentration. Thus aggregation increases with increase in the AOT concentration.

Recently Schenkel and Kitchener (3) studied the aggregation in water of 10 μ diameter polystyrene spheres, the surfaces of which were fully sulfonated chemically (therefore not unlike our system electrically). They found that aggregation (increased sedimentation rate) became important when the added potassium nitrate concentration exceeded about 3×10^{-4} M. Aggregation increased to about 4×10^{-3} M salt. These investigators point out that this behavior is in accordance with the theoretical predictions of Verwey and Overbeek (6) that coarse particles (micron range) should exhibit a secondary minimum of about 6 kT deep in 1×10^{-4} M one-to-one electrolyte. These electrolyte concentrations are approximately in the same range as the Na^+ concentration at which aggregation begins in our hexadecane-water system. In our studies, the Na^+ concentrations were actually a little greater, *viz.*, about 2 to 4×10^{-3} M in Na^+. So, if anything, the conditions in our systems were actually somewhat more conducive to aggregation. Perhaps a smaller London constant is appropriate for the hexadecane-water system. This may account for the difference.

The above discussion shows that it is very likely that the aggregation phenomenon may be basically an electrically induced one and that it appears to be unnecessary to postulate film-film attraction. It is quite possible that both mechanisms are important, since increasing ionic strength invariably increases the tendency for the aggregation of the surfactant itself. More basic studies are needed to provide definite answers to this problem.

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Application of Absorbancy Ratios to the Analysis of Pharmaceuticals IV

Analysis of Ternary Mixtures

By M. PERNAROWSKI†, A. M. KNEVEL, and J. E. CHRISTIAN

Four equations that may be used in the analysis of ternary mixtures are derived. All of these equations are based on the relationship between absorbancy ratio values and the relative concentration of one of the components in the ternary mixture. To indicate the applicability of these equations, three ternary mixtures were investigated, one in the ultraviolet region and two in the infrared region of the electromagnetic spectrum. Methods of plotting data are discussed and the utility of such plots under specific analytical situations are pointed out.

PREVIOUS PAPERS in this series dealt with the analysis of binary and certain types of ternary mixtures by the absorbancy ratio method of analysis (1–3). Comprehensive derivations and a survey of the literature may be found in the original work of the author (4). For the purposes of this paper, Q value implies an abbreviation for absorbancy ratio value. Similarly, the absorbancy ratio value obtained by, for example, dividing the absorbancy observed at 250 mμ by that observed at 275 mμ, the solute and the solution being the same for both measurements, is symbolized as $Q{:}250{:}275$. A Q curve illustrates the relationship between absorbancy ratio values and the relative concentration of binary or ternary mixtures. Other terminology and symbology shall be that suggested by the National Bureau of Standards (5). The symbol for cell length (b) shall not be used in the derivations below since the value is assumed to be equal to unity.

THEORY

When the three components of a ternary mixture have individual spectral characteristics such that each interferes during analysis with the other two at all wavelengths, then the equations derived below may be used for the simultaneous determination of the substances. As in most of the derivations in this series, it is assumed that two of the three components in the mixture have a characteristic isoabsorptive point. Figure 1 illustrates the type of spectral characteristics suited to the equations below.

The total absorbancy at λ_5 (A_5) is equal to the sum of the absorbancies due to X, Y, and Z. The following equation is, therefore, valid

$$A_5 = a_7\,Cx + a_5\,Cy + a_1\,Cz \qquad \text{(Eq. 1)}$$

Cx, Cy, and Cz are the concentrations of X, Y, and Z in the mixture. Preceding each of these symbols are the absorbancy index values for the three components at this wavelength. For exactly the same reasons

$$A_4 = a_6\,Cx + a_6\,Cy + a_2\,Cz \qquad \text{(Eq. 2)}$$

Continuing the derivation in the same way as indicated in the first paper in this series (1), Eq. 1 is divided by Eq. 2. The concentration terms are converted to fractional quantities by dividing each term of the equation by $Cx + Cy + Cz$. This results in the following equation

$$\frac{A_5}{A_4} = \frac{a_7\,Fx + a_5\,Fy + a_1\,Fz}{a_6\,(Fx + Fy) + a_2\,Fz} \qquad \text{(Eq. 3)}$$

However

$$Fy = 1 - Fz - Fx \qquad \text{(Eq. 4)}$$

The right-hand member of Eq. 4 is now substituted for Fy in Eq. 3 and the terms are collected. Each term is now divided by a_6.

$$\frac{A_5}{A_4} = \frac{\dfrac{a_7\,Fx}{a_6} + \dfrac{a_5\,(1 - Fz - Fx)}{a_6} + \dfrac{a_1\,Fz}{a_6}}{\dfrac{a_6\,(1 - Fz)}{a_6} + \dfrac{a_2\,Fz}{a_6}} \qquad \text{(Eq. 5)}$$

The spectral characteristics in Fig. 1 indicate that a_7/a_6 is the absorbancy ratio value for pure X (Qx) at these two wavelengths. Similarly, a_5/a_6 is the absorbancy ratio value for pure Y (Qy). The absorbancy ratio value for the mixture is equal to A_5/A_4 and may be designated as Qo. These symbols are substituted into Eq. 5 and the equation is rearranged

$$Qo = \frac{Fx(Qx - Qy) + Fz\,(a_1/a_6 - Qy) + Qy}{1 + Fz\,(a_2/a_6 - 1)} \qquad \text{(Eq. 6)}$$

Equation 6 contains two unknowns and, therefore, cannot be used to determine the components in the mixture. Another equation is required to complete the analysis. This equation may be derived from the spectral characteristics of the three components at λ_3 and λ_4 in exactly the same manner as shown above. Therefore

$$Qo_1 = \frac{Fx\,(Qx_1 - Qy_1) + Fz\,(a_3/a_6 - Qy_1) + Qy_1}{1 + Fz\,(a_2/a_6 - 1)} \qquad \text{(Eq. 7)}$$

Received June 3, 1961 from the School of Pharmacy, Purdue University, West Lafayette, Ind.
Accepted for publication September 21, 1961.
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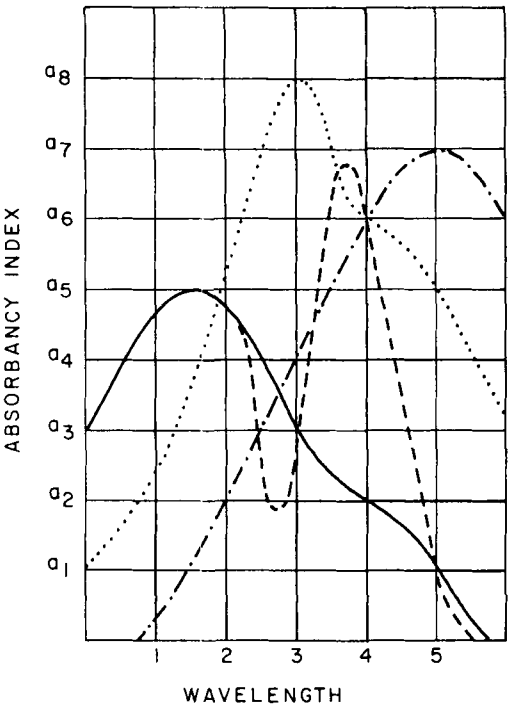


Fig. 1.—Hypothetical spectrophotometric curves of X (—·—·—), Y (. . . . .), and Z (——) illustrating spectral characteristics necessary for ternary Q analysis. The spectrum for Z (– – –) illustrating a common isoabsorptive point is also shown.

Equations 6 and 7 may now be solved simultaneously for the fraction of two of the three components in the mixture.

The above equations are simplified considerably if the three components in the mixture have a common isoabsorptive point. The total absorbancies at λ_3 and λ_4 may be defined as above except that the absorbancy index value of component Z at λ_4 is equal to a_5. By carrying out the derivation in exactly the same manner as above, Eqs. 6 and 7 become

$$Qo = Fx\,(Qx - Qy) + Fz\,(Qz - Qy) + Qy \quad \text{(Eq. 8)}$$

and

$$Qo_1 = Fx\,(Qx_1 - Qy_1) + Fz\,(Qz_1 - Qy_1) + Qy_1 \quad \text{(Eq. 9)}$$

These two equations may now be solved simultaneously for the fractions of X and Z in the mixture.

The disadvantages of this type of analysis are apparent from the above equations. Two simultaneous equations must be solved in order to complete the analysis. This, however, is somewhat simpler than the solution of three equations simultaneously as would normally be required for a three-component mixture. The major disadvantage occurs when there is no common isoabsorptive point in the spectra of the three components. When this occurs, absorbancy index values at three wavelengths for one of the three components are required to apply the above equations.

The first paper in this series (1) showed that a plot of the absorbancy ratio values of binary mixtures *vs.* the relative concentration of such mixtures was a straight line if one of the two wavelengths used in the analysis represented an isoabsorptive point. A similar situation holds for ternary mixtures. If the fraction of one of the components in a ternary mixture is held constant, a plot of absorbancy ratio *vs.* the fraction of one of the other two components results in a straight line. An examination of Eq. 6 will indicate the reason for this. With Fz a constant, this equation assumes the form of $y = mx + b$. The slope and intercept of this line will depend on the fraction of Z in the mixture. If a series of solutions are prepared, each series containing a fixed quantity of Z and varying quantities of X and Y, and the absorbancy ratios derived from these plotted *vs.* the relative concentration of either X or Y in the mixture, a series of straight lines will result. These lines will intersect at some point either on the graph or at an extrapolated distance from it.

A similar condition holds for Eq. 8. In this case, however, a series of straight lines will result which have the same slope but different intercepts. This occurs because of the isoabsorptive point at λ_4. The analytical use of this type of plot and of that above will be discussed in more detail below. The most important consideration here is that if independent analysis of Z is possible by other means, the above equations, based on the principles of Q analysis laid down in earlier publications (1–3) may be used to analyze for the other two components in the mixture and hence obviate the necessity of solving simultaneous equations.

EXPERIMENTAL

The apparatus and general techniques used in the analysis of the ternary systems described below have been reported in previous publications (1, 2). Two ternary systems were analyzed in order to show the validity of the above equations.

Analysis of Mixtures Containing Phenyl Salicylate, Salicylic Acid, and Gentisic Acid.—These three substances are not appreciably soluble in water. Stock solutions, therefore, were prepared with the aid of alcohol. Since the latter two substances under investigation are acidic, the spectral characteristics can be expected to vary with the pH of the solution being studied. All solutions, therefore, were prepared in such a way that each contained 1.00% concentrated hydrochloric acid in the final dilution. The spectrophotometric curves for these three substances are shown in Fig. 2. Salicylic acid, phenyl salicylate, and gentisic acid absorb ultraviolet radiant energy most strongly at 303, 307, and 330 mμ, respectively. Minima occur at 272, 260, and 270 mμ for phenyl salicylate, salicylic acid, and gentisic acid, respectively. An isobsorptive point for these three substances occurs at 314 mμ.

Three wavelengths, 303 mμ (the peak for salicylic acid), 314 mμ (the isoabsorptive point), and 330 mμ (the peak for gentisic acid) were chosen for the analysis. Q:303:314 and Q:330:314 values for these three substances were determined and are reported in Table I.

It was pointed out above that a series of straight lines result when the absorbancy ratio is plotted *vs.* the per cent of one of the components in the mixture

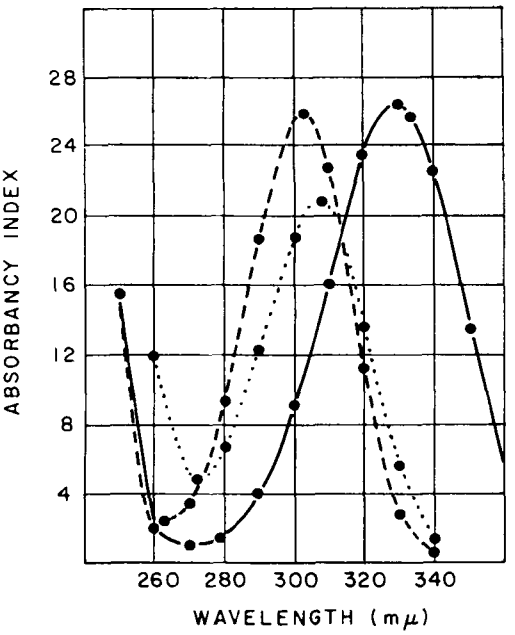


Fig. 2.—Spectrophotometric curves for phenyl salicylate (. . . . .), salicylic acid (– – – –), and gentisic acid (——) in 0.1 *N* hydrochloric acid.

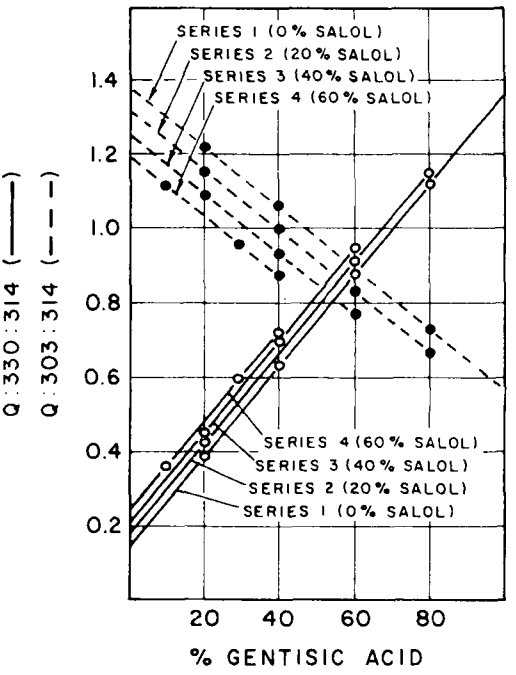


Fig. 3.—*Q* curves for gentisic acid, salicylic acid, and phenyl salicylate (salol).

if one of the three components in such a mixture is held constant. Four series of solutions were so prepared that the first contained 0% phenyl salicylate, the second 20% phenyl salicylate, the third 40% phenyl salicylate, and the fourth 60% phenyl salicylate. The amounts of salicylic acid and gentisic acid within a series were allowed to vary. For any particular series, therefore, the amount of phenyl salicylate is a constant. The variables in the series are the quantities of gentisic acid and salicylic acid. *Q*:330:314 and *Q*:303:314 values were determined for all solutions and the results plotted in the usual way. These results are shown graphically in Fig. 3 and the equations for these lines are indicated by the data tabulated in Table II. Within experimental limitations, the slopes of these lines are equal. Hence, the lines are parallel. The intercepts of the lines differ, indicating the validity of Eqs. 8 and 9. These equations may now be rewritten in their numerical form. All slope values (that is, the difference between two *Q* values as shown in Eqs. 8 and 9) were determined by the method of least squares from data accumulated on mixtures. There is, therefore, a slight difference between these values and those obtained from the data in Table I. As stated in previous papers (1, 2), the method of least squares gives a more accurate estimate of the slope and hence such values are carried into future calculations

$$Q{:}330{:}314 = 1.224\ Fx + 0.141\ Fz + 0.143$$

and

$$Q{:}303{:}314 = -0.809\ Fx - 0.318\ Fz + 1.383$$

Fx and *Fz* represent the fractional quantities of gentisic acid and phenyl salicylate, respectively. Experimentally, absorbancies are measured at the three wavelengths indicated above, the absorbancy ratio values calculated, and the above two equations solved simultaneously to determine the quantity of gentisic acid and phenyl salicylate in the mixture. The amount of salicylic acid is obtained by subtracting the sum of the fractional quantities of these two components from 1.

Five solutions containing these three substances were prepared and analyzed. The results obtained are shown in Table III.

Simultaneous Analysis of Quinine, Brucine, and Strychnine.—The simultaneous analysis of brucine and strychnine by the absorbancy ratio technique was described in a previous publication (2). If the alkaloid quinine is added to such a binary mixture, the resulting ternary mixture can be analyzed by

Table I.—*Q* Values for Phenyl Salicylate, Salicylic Acid, and Gentisic Acid[a]

Compound	*Q*:330:314	*Q*:303:314
Phenyl salicylate	0.282 ± 0.004	1.071 ± 0.002
Salicylic acid	0.143 ± 0.001	1.383 ± 0.006
Gentisic acid	1.367 ± 0.004	0.571 ± 0.004

[a] Each *Q* value is the mean of five determined values the standard deviation is reported after each value.

Table II.—Equations of *Q* Curves Shown in Fig. 3

Series	% Phenyl Salicylate in Mixtures	Line *Q*:330:314 *vs.* % Gentisic Acid Slope	Intercept	Line *Q*:303:314 *vs.* % Gentisic Acid Slope	Intercept
1	0	0.0122	0.148	−0.00809	1.380
2	20	0.0122	0.174	−0.00822	1.324
3	40	0.0124	0.203	−0.00807	1.255
4	60	0.0125	0.224	−0.00823	1.199

TABLE III.—RESULTS OF THE ANALYSIS OF MIXTURES CONTAINING GENTISIC ACID, SALICYLIC ACID, AND PHENYL SALICYLATE

Solution	Gentisic Acid		Salicylic Acid		Phenyl Salicylate	
	Present, %	Found, %	Present, %	Found, %	Present, %	Found, %
1	20.0	20.5	60.0	60.4	20.0	19.1
2	10.0	9.9	30.0	31.2	60.0	58.9
3	40.0	40.2	60.0	59.8	0.0	0.0
4	20.0	20.2	40.0	39.4	40.0	40.4
5	0.0	0.1	40.0	41.7	60.0	58.2

TABLE IV.—EQUATIONS OF *Q* CURVES SHOWN IN FIG. 4

Series	% Quinine in Mixture	Line *Q*:7.78:6.83 *vs.* % Brucine Slope	Intercept
1	0	1.096	0.612
2	25	1.269	0.625
3	50	1.491	0.626
4	70	1.564	0.623

applying the principles inherent in Eq. 6. This, however, presupposes that the cinchona alkaloid can be determined at some wavelength at which brucine and strychnine transmit all or nearly all of the radiant energy passing through the solution.

Infrared spectra of solutions of quinine (0.5 to 2.0 Gm. quinine per 100 ml. of chloroform) were compared with spectra of brucine and strychnine and, on this basis, it was apparent that quinine can be analyzed in the presence of these two substances by measuring absorbancies at 6.13 μ. Solutions of quinine in chloroform were first considered. A linear relationship was observed between the absorbancy at 6.13 μ and the concentration of quinine (C_q) in the solution expressed in terms of Gm. per 100 ml. The equation of this line was found to be: $A_{6.13\,\mu} = 0.283\ Cq + 0.025$. It should be pointed out that th s equation is valid only for the conditions of operation cited earlier (2). The intercept of the above equation indicates that there is a slight deviation from Beer's law at this wavelength. To avoid gross errors in the analysis, this equation was used to calculate Cq for all determinations at this wavelength.

Equation 6 presupposes a knowledge of absorbancy index values for quinine at the two wavelengths at which brucine and strychnine are analyzed (6.83 and 7.78 μ). In a manner similar to that described above, the absorbancies of solutions of quinine at these two wavelengths were plotted *vs.* the concentration (Cq) in Gm. per 100 ml. of chloroform. The equations of the two straight lines are: $A_{6.83\,\mu} = 0.173\ Cq$ and $A_{7.78\,\mu} = 0.095\ C_q + 0.004$. Both equations indicate that Beer's law is obeyed at these wavelengths.

The inherent qualities of Eq. 6 were discussed above. To check the validity of these observations in the infrared region of the spectrum, four series of solutions were prepared in a manner analogous to that described under the section on the analysis of phenyl salicylate, salicylic and gentisic acids. *Q*:7.78:6.83 values were determined for each of the solutions within a series and the results plotted in the usual way. These results are shown graphically in Fig. 4 and in tabular form in Table IV. This table shows the slopes to be different and the intercepts the same. This, however, is a rather unique case. It was pointed out above that there should

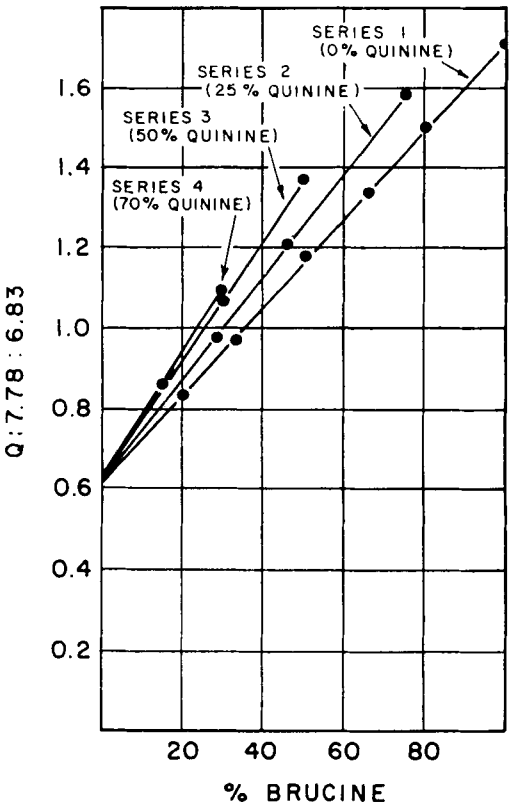


Fig. 4.—*Q* curves for brucine, strychnine, and quinine.

be a point of intersection in lines such as these. In this case, this point occurs at or near the intercept. In other ternary mixtures, however, this would not be the case. The *Q* value at the point of intersection would not necessarily coincide with the intercept value.

On the basis of the data reported here or in a previous publication (2), Eq. 6 may be rewritten as follows

$$Q{:}7.78{:}6.83 = \frac{1.096\ Fb + Fq\ (0.095/0.371 - 0.612) + 0.612}{1 + Fq\ (0.173/0.371 - 1)}$$

Fb is the fraction of brucine in the ternary mixture and *Fq* the fraction of quinine in the same mixture. Experimentally, the numerical value of *Fq* is obtained by determining the absorbancy of the mixture at 6.13 μ, applying the necessary correction factors cited above, and dividing the value obtained for Cq by weight of the sample taken in the analysis.

TABLE V.—RESULTS OF THE ANALYSIS OF MIXTURES CONTAINING QUININE, BRUCINE, AND STRYCHNINE

Solution	Quinine		Brucine		Strychnine	
	Present, %	Found, %	Present, %	Found, %	Present, %	Found, %
1	50.0	47.0	30.0	31.9	20.0	21.1
2	25.0	24.2	28.7	29.4	46.3	46.4
3	0.0	0.0	80.1	80.8	19.9	19.2
4	40.1	41.3	9.6	10.1	50.3	48.6
5	50.0	51.6	50.0	51.6	0.0	0.0

This value is now substituted into the above equation, the Q:7.78:6.83 value calculated from the absorbancies observed at 7.78 and 6.83 μ, and the fraction of brucine in the mixture calculated by solving this equation. The fraction of strychnine in the mixture can now be determined by subtracting the sum of Fb and Fq from 1.

A number of solutions containing quinine, brucine, and strychnine were prepared and analyzed. The results obtained for five of these solutions are shown in Table V.

DISCUSSION

The absorptiometric principles involved in the analysis of ternary mixtures do not differ from those for binary mixtures. While the equations derived appear to be more complicated, they are still based on Beer's law and the principles of absorbancy ratio analysis outlined in previous publications (1, 2). The comments in these two publications are as applicable here as they were to the analysis of binary systems. The pertinent factors cited therein included slope of the Q curve, accuracy of the absorbancy measurement, relative concentrations of the active ingredients, nature of the pharmaceutical, and spectral characteristics of the components of the mixture.

Several points arise, however, from the experimental data accumulated on ternary mixtures. It was pointed out above that a straight line results if the Q values of ternary mixtures in which one of the components is held constant are plotted *vs.* the relative concentration of one of the components in the mixture. This is shown graphically in Figs. 3 and 4. The question arises as to the utility of such plots. Three possible situations can result from such plots. The first involves the case where all three components have an isoabsorptive point in common, the second where only two of the three components in the mixture have a common isoabsorptive point, and the third where no suitable isoabsorptive point occurs.

If the absorbancy ratio values of ternary mixtures and the relative concentrations are compared in the usual way, and only two of the three components share an isoabsorptive point, then a point arises in the series of curves plotted at which all mixtures have the same absorbancy ratio value. Such a point may represent experimental or hypothetical mixtures. In the case of quinine, brucine, and strychnine, the point at which all lines intersect represented an experimental condition. It should be evident that such a point could occur (on extrapolation) to the left of the Y axis if a different ternary mixture were investigated. The utility of such a point of intersection is not difficult to ascertain. In the vicinity of such a point, an analysis of the ternary mixture by the principles laid down herein would yield poor analytical results. This occurs simply because any number of mixtures have the same Q value. This then indicates that the presence of such a point on the graph is not desirable if the entire range of the mixture (0 to 100%) is to be investigated. It is important to consider, therefore, the relative concentrations of the three components in the mixture. If these are such that Q values obtained on analysis are sufficiently removed from this point of intersection, then the analysis will yield satisfactory results. For example, a 1-1-1 mixture might be easily analyzed, but it would be impossible to analyze accurately a 3-2-1 mixture. By plotting results in the manner shown in Fig. 4, the feasibility of the analysis can be quickly established.

A somewhat different situation occurs in the case of phenyl salicylate, salicylic and gentisic acids. These three substances have a common isoabsorptive point and hence the slope of the Q curves is a fixed quantity. This value is always equal to the slope of the Q curve for the binary mixture composed of two of the three substances in the ternary mixture. The absorbancy index value for the third component at the first wavelength used for the calculation of the Q values is, therefore, the significant constant. If the value of a_1/a_6 approaches that of Qy (see Eq. 6), then the analysis will not be very accurate primarily because the third component is not altering the intercept value of the Q curves to a significant extent. This results in a situation where many mixtures have essentially the same Q value. If a_1/a_6 and Q_y differ drastically (but again not so drastically that their determination becomes difficult) then the analysis can be expected to yield results in keeping with good analytical practice.

To summarize, therefore, a series of Q curves for ternary mixtures gives the analyst an indication of the accuracy with which the analysis can be conducted. If a point of intersection is present on the graph, the analyst can expect that analysis of mixtures having compositions defined by the graph in the vicinity of this point will not be accurate. The greater the difference between a_1/a_6 and Qy (on the basis of Eq. 6) the better the results will be.

The reader may well ask what would happen if no suitable isoabsorptive point is present in the spectra of two of the three substances. This obviously complicates the equations derived in this paper but, again, if one of the components is held constant, a plot of absorbancy ratio *vs.* the per cent of one of the other components in the ternary mixture should result in a curve. In the second paper in this series (2), the potassium bromide technique was used in the analysis of two sulfa drugs. This binary mixture can, in fact, be considered as a ternary mixture containing potassium bromide, sulfathiazole,

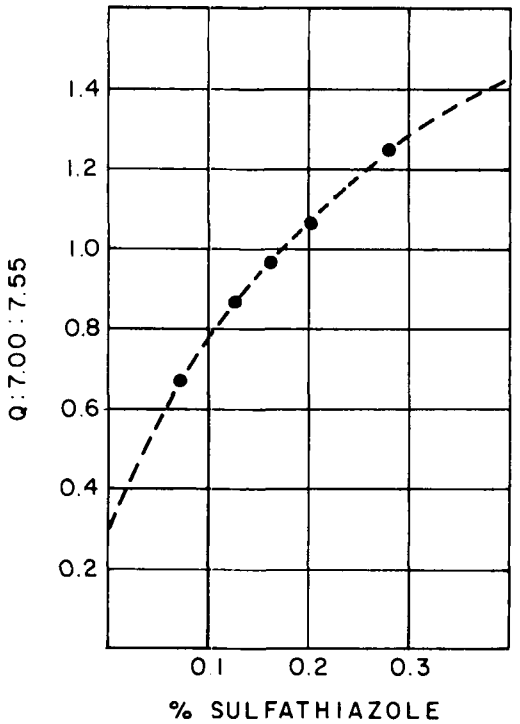


Fig. 5.—Q curve for sulfathiazole, sulfapyridine, and potassium bromide.

and sulfapyridine. Potassium bromide has linear but significant absorption (as mentioned in the earlier paper) but is also present in what might be considered as constant quantities. The pellets prepared for this investigation contained very near to 99.6% potassium bromide and 0.4% active ingredient or ingredients. Since it was only the latter value that was representative of the two variables, the 99.6% of potassium bromide was a constant. It was mentioned in the earlier publication that neither of the two wavelengths (7.00 and 7.55 μ) used in the analysis represented an isoabsorptive point. A plot of Q:7.00:7.55 *vs.* the % of sulfathiazole in a ternary mixture containing the components under discussion should result in a curve. This is the case and such a curve is shown in Fig. 5. Once such a curve is constructed, it can be used to determine the concentration of sulfathiazole (and sulfapyridine, by proper choice of wavelengths) in unknown mixtures.

The major disadvantage of this type of analysis is the need for a simultaneous solution of two equations. The constants required for these equations are relatively easy to accumulate primarily because most of these are absorbancy ratio values and hence concentration independent. Equation 6 or its equivalent does provide an alternative approach. If the third substance in a mixture can be determined by some other method of analysis in such a way that the other two components do not interfere, then the other two components in the ternary mixture can be resolved by application of the above equation. Such situations often arise in pharmaceutical analysis. If one of the three components in the mixture is acidic, for example, then a volumetric method of analysis might be used to determine its concentration. From a knowledge of the initial weight of the sample, the fraction of this acidic substance in the mixture can be determined. Equation 6 will now yield relative values for the other two components. These, however, can be easily converted to absolute values on the basis of the weight of the sample taken in the analysis.

The accuracy of this technique does not approach that found in the case of binary mixtures. The ternary mixtures investigated could be analyzed to within approximately 2 to 3% of theory. Since this presupposes no separation of the components, the accuracy is probably better than that obtained by more conventional techniques.

CONCLUSION

A number of equations have been derived to show the applicability of the absorbancy ratio technique to the analysis of ternary mixtures. Such mixtures can be resolved to within 2 to 3% of theory provided the spectral characteristics of the individual components meet the criteria listed above. Other limitations of this technique are discussed.

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Free Radical Studies by Electron-Spin Resonance of Some Derivatives of Phenothiazine

By DAVID W. SCHIESER† and L. DALLAS TUCK

The existence of stable free-radical semiquinoid intermediates in the oxidation of several N-substituted phenothiazine derivatives of therapeutic importance has been demonstrated by means of electron-spin resonance. The spectra differ from those of the parent compound in the lack of hyperfine structure.

BY MEANS of electron-spin resonance, stable semiquinone free radicals have been shown to be formed upon oxidation of certain N-substituted derivatives of phenothiazine. This is an extension of work reported elsewhere (1) on phenoxazine, phenothiazine, and some ring-substituted derivatives of the latter. The compounds of the present study, which are of medical importance because of their tranquilizing, antihistaminic, and antinauseant effects, are (I) prochlorperazine, (II) chlorpromazine, (III) trifluprom azine, and (IV) perphanazine. As is seen by their structural formulas in Fig. 1, compounds I and IV contain the common structure of an *n*-propyl chain on the nitrogen, terminated by a piperazine ring, and differing from each other by the substitution in the 4-position. Compounds II and III contain a dimethylaminopropyl chain on the nitrogen, and differ from each other by the nature of the substituent β to the nitrogen.

EXPERIMENTAL

The spectrometer used in this research is described elsewhere (1). Measurements were made as the derivative of the absorption at a frequency of 9400 megacycles per second and at a magnetic field strength of approximately 3300 gauss.

All of the free radical solutions were prepared by dissolving approximately 100 mg. of the solid parent compound in 10 ml. of concentrated sulfuric acid. In all cases but one, it was found that the sulfuric acid was able to oxidize rapidly the drugs to the free radical state. In the case of trifluprom azine, the free radical was produced only after addition of 0.1 ml. of 30% hydrogen peroxide to the acid solution.

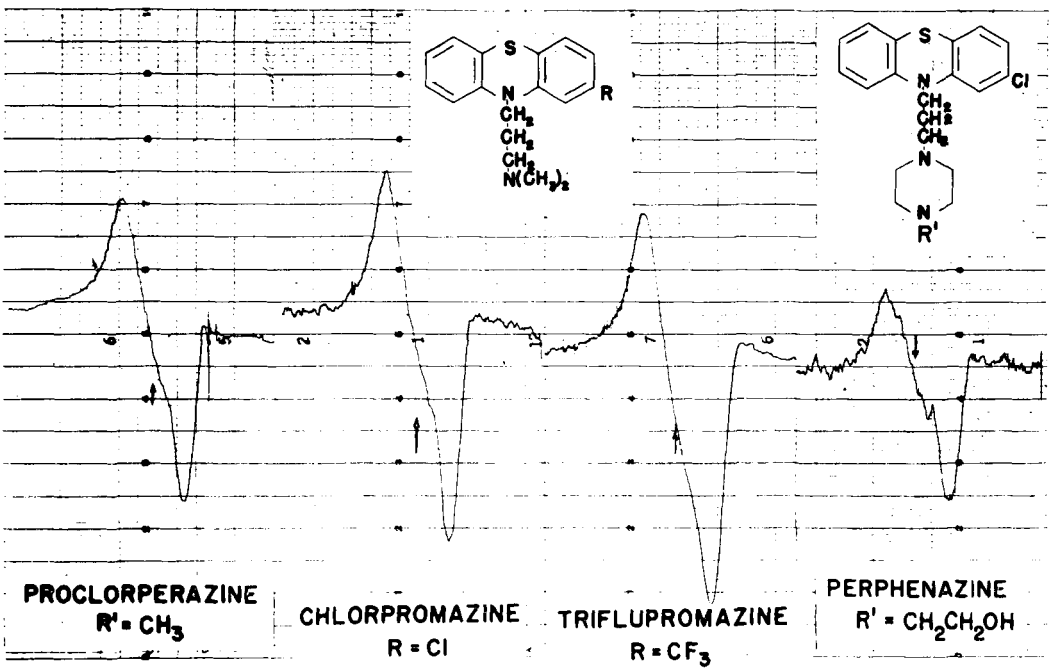


Fig. 1.—Electron-spin resonance spectra of free radicals formed by oxidation of prochlorperazine, chlorpromazine, trifluprom azine, and perphenazine. Each horizontal division is 7.4 gauss. Low magnetic field is to the right on this figure.

Received November 25, 1960, from the School of Pharmacy, University of California, San Francisco 22.
Accepted for publication October 6, 1961.
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RESULTS AND DISCUSSION

Figure 1 shows the spectra of the four free radicals and the structural formulas of the parent compounds (reduced state). Table I presents their *g* values

TABLE I.—g VALUES OF FOUR FREE RADICALS

	g Value
Prochlorperazine	2.0036
Chlorpromazine	2.0034
Triflupromazine	2.0034
Perphenazine	2.0029

determined by comparison with diphenylpicryl hydrazyl, whose resonant position in the magnetic field is indicated in each spectrum by an arrow.

It is seen that the nature of the substituent has almost no perceptible effect on the appearance of the spectrum and has only a minor effect on the *g* value. These spectra appear to consist of a single absorption line, different from that of phenothiazine and its ring-substituted derivatives, all of which give a spectrum of four well-resolved lines. The latter spectra have been interpreted in terms of the hyperfine splitting by the nitrogen nucleus together with the proton on this position in acid solution. In each of the present spectra, the slight inflection point suggests that the spectrum is the summation of three equally weighted broad component lines arising from splitting by the nitrogen nucleus. The breadth of the lines in this degree appears to be due to the hyperfine splitting by the protons in the side rings and in the complex chain attached to the nitrogen. Other contributory factors to the line breadth are the nature of the solvent system and the magnetic dipole-dipole interaction from neighboring molecules. However, these factors must not play an important role in the present case, as the conditions were comparable with those under which four well-resolved lines were obtained with phenothiazine and other compounds.

Further discussion of these effects will appear in a subsequent publication in which a series of N-alkyl-substituted radicals are studied (2).

Further work is planned to ascertain the concentration of free radical under various conditions of solvent and acidity, and therefrom the semiquinone formation constants (dismutation constants) will be computed.

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Simplified Method for the Measurement of Actomyosin Contraction

By DUANE G. WENZEL and HARBANS LAL

ACTOMYOSIN, the principal contractile protein in muscle, exists in the form of contractile filaments. It is possible to extract these filaments, fragmented transversely but otherwise undamaged, from muscle fiber. The microscopic filaments may then be realigned into a three-dimensional network capable of sustaining tension. It was demonstrated some years ago that threads formed from this actomyosin responded to the addition of adenosine triphosphate (ATP) with spectacular shortening (1). These first threads, however, were composed of poorly oriented actomyosin filaments and did not develop any appreciable tension (2). Close packing of the filaments by treatment with glycerol, drying, and stretching produced threads capable of developing tension (3). It was later demonstrated that relatively strong fibers could be formed by lateral compression of surface-spread layers of actomyosin (4).

The actomyosin model is similar, in many respects, to its intact muscle counterpart. Histologically, of course, the original muscle fiber also possesses a sheath or sarcolemma. The response of the model to ATP is, for all practical purposes, the same as that of the muscle fiber. Because of the relative simplicity of this system and because a specific muscle component may be studied independently, the actomyosin threads or fibers have become popular models for the study of muscle pharmacology.

Received August 17, 1961, from the University of Kansas, School of Pharmacy, Lawrence.
Accepted for publication September 7, 1961.

A number of methods have been employed to measure the contractile response of actomyosin. A simple procedure introduced by Hayashi (5) and later modified by Robb, *et al.* (6), employs a vertical fiber supporting a glass weight. This fiber was prepared in a Langmuir trough and then transferred to a beaker containing the bath solution. The response of the system was determined by measuring the movement of the weight with a microscope having a calibrated eyepiece. This method has several disadvantages. First, the actomyosin fiber must be transferred from the trough to the contraction chamber and must further be extensively manipulated in order to be loaded. Second, the vertical position of the fiber can affect the response to ATP through variable buoyancy and the weight of the thread itself.

In an attempt to eliminate the above disadvantages, a new method for the preparation of the actomyosin fiber and a new measuring apparatus were introduced (7). In this method the surface film of actomyosin is not completely compressed to a thick fiber, but instead is formed into a wider band of a constant width and minimal thickness. This assures a uniform diffusion of ATP into the center of threads or fibers which are still thick enough to support a weight. It has been suggested that during the isotonic contraction of actomyosin, the "unplasticized" core of the fiber not reached by ATP may interfere with the shortening (8). This method utilizing the wide band of actomyosin also permits horizontal attachment of the band between a fixed support extending from the bottom of the

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trough and an elongated arm of a 500-mg. capacity Roller-Smith torsion balance. Comparison of the results obtained using vertical fibers to those using horizontal bands indicates a definite reduction in the scatter of observation with the latter method (7). Loading of the band is accomplished by moving the balance arm to the desired number of milligrams. Contraction of the actomyosin band then initiates an electromechanical feed-back mechanism causing the trough to move in a direction equal to and opposite to that of the contraction. Movements of the trough are recorded with an oscillograph after suitable amplification.

The purpose of the present study was to determine the feasibility of measuring the contraction of horizontal actomyosin bands with a microscope having a calibrated eyepiece.

EXPERIMENTAL

The apparatus employed consisted of three major components. A Langmuir trough of methyl methacrylate polymer,[1] similar to that of Bing and co-workers, was employed (7). The inner dimensions were 48 × 12.5 × 1.75 cm. A separate chamber in which the band contracts was formed by the addition of an inner wall about half the height of the trough. This chamber had the dimensions of 12.5 × 3.8 × 0.9 cm. A series of holes, 3 mm. in diameter and 0.5 cm. apart, were bored along the longitudinal axis of this chamber. These served to fix a small plastic[1] block, 6 mm. in height, which was fitted with a peg to be plugged into one of the holes. The trough was mounted on a platform fixed to a small scissors-type auto jack which was used to raise the chamber into position beneath the balance arm. A Roller-Smith torsion balance with a 500-mg. capacity was fastened on a rack over the trough, as shown in Fig. 1. The weighing pan of the balance was replaced with an aluminum spatula which extended vertically over the contraction chamber. The flat blade of the spatula was bent at a right angle to the shaft in order to provide support for the actomyosin band. Measurement of the movement of the band was accomplished with a stereoscopic microscope as described by Robb and Mallov (6). The system employed thus combines the simplicity of the visual method for microscopically measuring movement of the actomyosin fiber with the improved horizontal loaded actomyosin band.

Actomyosin was extracted from fresh dog heart in the usual manner and a band was formed in the trough as previously reported (7). This band was attached to the block fixed in the base of the bath and to the movable balance arm. A load of 10 mg. was placed on the band by movement of the weighing spring of the torsion balance. The zero point of the calibrated microscope eyepiece was then focused on the edge of the movable arm. Ten milliliters of the chamber solution were removed and replaced with a concentrated solution of the sodium salt of ATP (sigma) to make a final concentration of 0.005 *M*. Measurements were recorded at 2, 4, 8, 16, and 32 minutes following addition of the ATP. Five bands prepared from freshly extracted actomyosin were tested on the same day. The per cent shortening from the original length was determined for each band. Initial band lengths

[1]Tradename, Plexiglas.

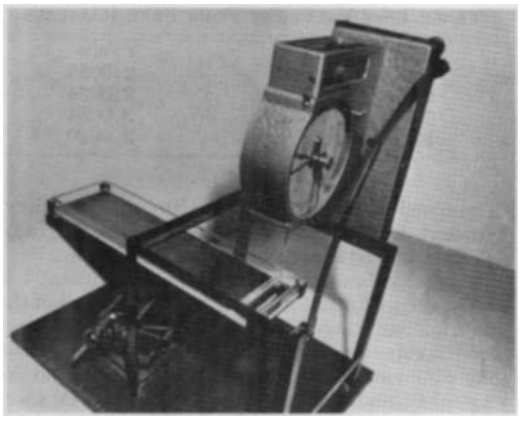


Fig. 1.—Apparatus for the measurement of actomyosin band contractions. Stereoscopic microscope used to perform actual measurements is not shown.

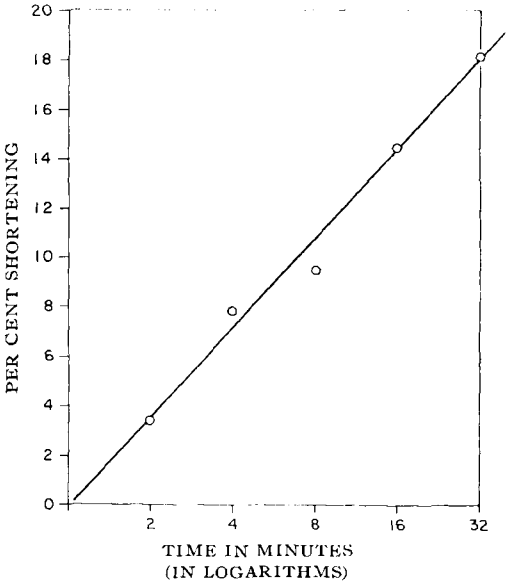


Fig. 2.—Mean per cent shortening of five actomyosin bands at several times.

for the five bands ranged between 20 and 25 mm. The mean per cent shortenings obtained with these five bands plotted against the logs of the times tested are shown in Fig. 2.

From the linearity of the response it is apparent that the method described can be employed to measure effectively the rate and extent of loaded, isotonic actomyosin contractions.

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Stabilization of Trypsin in Ointment Bases

By JESS A. BUSH†

Calcium has been used to stabilize trypsin in a polyethylene glycol ointment base. The samples were kept at room temperature and at 37° for a period of 6 months. Results of assays on these samples are reported. Eighty per cent of the trypsin activity was retained in the presence of calcium, including the samples stored at 37° for 6 months.

THE USE of proteolytic enzymes to remove necrotic tissue often present in burns, amputation stumps, wounds, and many other conditions is becoming more widespread. Incorporation of the enzyme into an ointment base provides a convenient method for use. Packaging is also simplified, since it is less expensive to put the preparation in one container than to package buffer and enzyme separately. An ointment is ready for immediate use, but when separate solutions are provided, the enzyme must be dissolved in the buffer before use.

The stabilization of the enzyme in the ointment base is of the utmost importance. Trypsin has been shown to be stable for several months in a Carbowax base ointment at 4° or less (1). However, at room temperatures proteolytic activity is lost. Calcium^{2+} (2, 3) has been used to stabilize trypsin. By incorporating calcium^{2+} into an ointment base, the proteolytic activity may be preserved for long periods of time at room temperature and at 37°.

EXPERIMENTAL

Ointments were prepared using polyethylene glycol ointment U.S.P. Fifty milligrams of noncrystalline trypsin isolated from beef pancreas was dissolved in one ml. of water and added to 27 Gm. of the ointment. This trypsin assayed 2000 Armour units/mg. Calcium chloride dihydrate was added in 1 ml. of water. After careful mixing of the ointment, the pH was adjusted to 6.7–6.8 using 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid. A pH meter was used for this adjustment. Buffers were not used because their influence on the stability of trypsin is not known. Water was added to bring the weight of each ointment to 30 Gm.

One set of ointments was stored at room temperature, another at 37°, and a third, which contained no calcium, at 3°. Table I lists the amount of calcium chloride dihydrate added and the proteolytic activity retained in the various ointments.

Assays were conducted using the hemoglobin method of Kunitz (4). For the assay of each sample, 2 levels of ointment were run in duplicate. Values given are the average per cent of proteolytic activity retained for each ointment after the time lapse indicated. Results agreed within ±10%. The reference standard was a highly purified crystalline trypsin which assayed 4000 Armour units/mg. It was standardized against tyrosine several times during the experimental work. The ointment kept in the refrigerator was used as the method control sample.

TABLE I.—PER CENT TRYPSIN ACTIVITY RETAINED

Temp., ° C.	Calcium Chloride Dihydrate, mg./30 Gm.	2 wk.	5 wk.	26 wk.
3	0.0	97.0	102.0	98.0
RT	0.0	80.0	71.0	63.0
RT	200.0	100.0	96.0	80.0
RT	400.0	100.0	99.0	83.0
37	0.0	81.0	51.0	32.0
37	200.0	95.0	80.0	80.0
37	400.0	95.0	85.0	82.0

Samples for assay were weighed, dissolved in water, and pipetted into the assay medium. A blank was prepared for both levels of each ointment by adding 5% trichloroacetic acid to the sample immediately after the ointment in water was added to the hemoglobin.

DISCUSSION AND SUMMARY

Results in Table I show that in the presence of calcium, trypsin losses are greatly reduced. There are slightly lower losses with the higher level of calcium, but the method of assay is not accurate enough to make this significant. However, there is definitely a difference between the samples containing calcium and those containing no calcium at both room temperature and at 37° after 5 weeks, or longer.

Twenty per cent loss after 6 months with calcium present is almost within the error of assay. The use of a 10–20% overage in the original preparation of the ointments would bring the results within labeled potency.

A method of stabilization of trypsin in an ointment base has been reported. It may be useful for further studies on stabilizing trypsin or other enzymes in ointments.

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Received September 6, 1958, from the School of Pharmacy, University of Southern California, Los Angeles and Southern California Gland Co., Los Angeles, Calif.
Accepted for publication December 8, 1961.
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Qualitative Color Test for Identification and Differentiation of Some Fluorinated Steroids

By E. IVASHKIV

A qualitative colorimetric test has been developed for the identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in pharmaceutical formulations. The test is based on a newly discovered reaction for fluorinated steroids and depends upon the formation of an amber color from fludrocortisone acetate, a yellow chromogen from hydrolyzed triamcinolone, and a pink color from triamcinolone acetonide by reaction of phenol, hydroquinone, and the phosphoric-sulfuric acid mixture.

MANY color reactions of steroids are used in qualitative tests to demonstrate the presence of these various steroids in pharmaceutical preparations. For example, *m*-dinitrobenzene-potassium hydroxide (1) and 2,6-di-*tert*-butyl-*p*-cresol (2) are useful identity tests for various fluorinated steroids.

This investigation is a study of the newly discovered color reaction for fluorinated steroids with phenol, hydroquinone, and the phosphoric-sulfuric acid mixture as a specific method of identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in pharmaceutical formulations.

EXPERIMENTAL

Method

Reagents and Equipment.—Sulfuric-phosphoric acid solution. To 850 ml. of concentrated sulfuric acid, 150 ml. of 85% phosphoric acid is added and mixed.

Phenol liquid, 85%, reagent grade.

Tetramethylammonium hydroxide solution, 10 ml. of 10% aqueous reagent is diluted to 100 ml. with ethanol.

Methylene chloride (dichloromethane), spectrophotometric grade.

Hydroquinone, purified.

Florisil, 60/100 mesh.

Purification column. The column consists of a glass tube of 10 mm. inside diameter and 150 mm. in length with a constricted end to retain the adsorbent. The column is packed with Florisil to the height of 40 mm. and is washed with 10 ml. of methylene chloride prior to use.

Test tubes, screw cap, 25 × 150 mm.

Unless specified, all chemicals are reagent grade.

Procedure

Sample Preparations.—*Creams, Lotions, and Ointments.*—A sample equivalent to 2 mg. of steroid is weighed into a test tube. Methylene chloride 20 ml., is added and the tube is covered with a plastic cap. The steroid is extracted by shaking for 2 minutes. Lotion samples are centrifuged at 1500 r.p.m. for 3 minutes. The upper, aqueous layer is discarded by means of an aspirator. Cream and ointment samples are filtered through No. 3 filter paper.

The methylene chloride solution is poured into a purification column. When the flow rate is slower than 75 drops per minute, it can be increased by the air pressure bulb fitted at the top of the column. The column is washed with 3 × 10 ml. of methylene chloride. The solvent is forced out by means of the rubber pressure bulb and the steroid is eluted with 10 ml. of methanol.

Suspensions and Sprays.—A sample is diluted with methanol to give the concentration of about 200 mcg. of steroid/ml.

Tablets.—A powdered sample equivalent to 4 mg. of triamcinolone or 1 mg. of 9α-fluorohydrocortisone is weighed into a test tube. Ethyl acetate, 10 ml. is added and the tube is covered with the plastic cap. Steroids are extracted by shaking for 3 minutes. The sample is filtered through No. 3 filter paper.

Color Development.—Aliquots of the sample extracts are placed in three test tubes designated A, B, and C. A 2-ml. portion of the methanolic sample or 1 ml. of the ethyl acetate tablet extract are the sample aliquots used. Glacial acetic acid (1 ml.) is added to tube A and the mixture is heated in a boiling water bath for 1 minute. Alcoholic tetramethylammonium hydroxide (1 ml.) is added to tube B and the tube is incubated at 50° for 15 minutes.

To all three tubes, 0.5 ml. of phenol, about 50 mg. of hydroquinone, and 5 ml. of sulfuric-phosphoric acid are added. The test tubes are mixed and heated in a boiling water bath for about 1 minute to speed up the color development.

A pink color in tubes A and C and yellow chromogens in tube B prove the presence of triamcinolone.

A pink color in all tubes indicates the presence of triamcinolone acetonide.

An amber color in tube A and orange-red color in tubes B and C indicate the presence of fludrocortisone acetate in a sample.

RESULTS AND DISCUSSION

The results summarized in Table I indicate that this method is useful for the identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in a variety of formulated products.

For good color development, the steroid tested has to be free from interfering substances. This is accomplished by centrifugation or filtration to remove insoluble matter and, if necessary, absorption on a Florisil column, followed by elution of the steroid fraction.

Methylene chloride was the solvent selected for the extraction of creams, lotions, and ointments because it appeared to be better than other solvents tested, both from the standpoint of extraction efficiency and the removal of impurities from the Florisil column. The steroids were eluted from the

Received July 1, 1961, from the Squibb Institute for Medical Research, New Brunswick, N. J.
Accepted for publication September 5, 1961.

TABLE I.—QUALITATIVE COLOR TEST OF STEROIDS

Name of Product	Type of Formulation	Developed Color[a] (Samples) A	B	C
Fludrocortisone acetate	Lotion	A	OR	OR
Fludrocortisone acetate, neomycin, gramicidin	Lotion	A	OR	OR
Triamcinolone acetonide	Lotion	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Lotion	P	P	P
Fludrocortisone acetate	Ointment	A	OR	OR
Fludrocortisone acetate, neomycin, gramicidin	Ointment	A	OR	OR
Fludrocortisone hemisuccinate, neomycin, gramicidin	Ointment	A	OR	OR
Triamcinolone acetonide	Ointment	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Ointment	P	P	P
Fludrocortisone acetate, neomycin, gramicidin	Cream	A	OR	OR
Triamcinolone acetonide	Cream	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Cream	P	P	P
Fludrocortisone hemisuccinate, neomycin, gramicidin	Suspension	A	OR	OR
Triamcinolone acetonide	Suspension	P	P	P
Triamcinolone acetonide	Spray	P	P	P
Fludrocortisone acetate	Tablet	A	OR	OR
Triamcinolone	Tablet	P	Y	P

[a] A, Amber; OR, orange-red; P, pink; Y, yellow.

column with methanol, but acetone or ether could also be used for this purpose. Dimethylformamide, an excellent solvent for the elution of steroids from Florisil columns, could not be used because it gives a yellow color with the steroids in the presence of tetramethylammonium hydroxide and sulfuric acid. Ethyl acetate was used for tablet extractions because no further purification of these extracts were required.

Either Florisil or aluminum oxide may be used as an adsorbent for fluorinated steroids. Florisil has been chosen in this method because of the easy removal of interfering substances from creams, lotions, and ointment formulations.

Triamcinolone and triamcinolone acetonide produce the same pink color in the presence of phenol, hydroquinone, and sulfuric-phosphoric acid. However, if a triamcinolone sample is heated in the presence of the alkaline solution at 50° for 15 minutes prior to the addition of reagents, yellow chromogens are obtained because triamcinolone is unstable in alkaline solutions at elevated temperatures. Experiments have shown that extensive degradation occurs at the temperature of 60° and above. Triamcinolone acetonide is slightly affected at 60° in the presence of tetramethylammonium hydroxide. Tetramethylammonium hydroxide has been chosen for the degradation purpose because it produces no heat after the addition of a sulfuric-phosphoric acid mixture. Heating fludrocortisone acetate at 100° in the presence of glacial acetic acid for 1 minute converts it to the free alcohol. Triamcinolone and triamcinolone acetonide are not affected when heated in the acid solution.

The color test was applied to various other steroids which are also used in pharmaceutical preparations. The results of these tests are given in Table II.

TABLE II.—COLOR PRODUCED WITH PHENOL, HYDROQUINONE, AND SULFURIC-PHOSPHORIC ACID

Name of Steroid	Developed Color
Cortisone acetate	Amber
Fluoxymesterone	Orange to red
Hydrocortisone	Orange to red
Prednisolone	Red to orange

The presence of both phenol and hydroquinone in the reaction mixture is very important. Either phenol or hydroquinone produces the same color, but a combination of both chemicals gives about twice the color intensity. About 50 mg. of hydroquinone and 0.5 ml. of phenol proved to be the most suitable for the color development. Sulfuric acid of at least 40% strength is needed for the reaction, but the 85% mixture gives the best results. An increase in the concentration of sulfuric acid speeds up the reaction, but concentrations above 85% give lower color intensities. Phosphoric acid is used for dilution purposes to keep the water content in the reaction mixture to a minimum.

The temperature is an important factor in the color development. An increase in temperature accelerates the degree of reaction, but considerable quantities of chromogens are destroyed when the reaction mixture is heated at high temperatures. Since the color development is more rapid at 100° this temperature has been chosen for completing the test in a minimum of time.

Although relatively large samples of steroids (2 to 4 mg.) have been used for analysis, it has been possible to detect as little as 10 γ/ml. of steroid in the final solution by this method.

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Photometric Microtitration of Catechols (Epinephrine)

By MURRAY M. TUCKERMAN

Catechols in a buffered aqueous solution may be quantitatively titrated with ferrous ethylenediamine sulfate standard solution. The photometric end point is determined at 530 mμ, the wavelength of maximum absorption for the purple complex formed between ferrous ion and catechols. Advantages over the U.S.P. assay for epinephrine are (*a*) sensitivity is increased and (*b*) an epinephrine reference standard is not needed.

IN THE ASSAY of dosage forms it is customary to seek a representative average sample by combining 20 or more dosage units. Occasionally, however, it is necessary to assay a single dosage unit or part of such a unit. As part of a project for the assay of single dosage units, a paper concerning anesthetics derived from aminobenzoic acid in cartridges for dental anesthesia was published in 1959 (1). The present paper proposes a method for assay of the catecholamine vasoconstrictor frequently present in such preparations.

The proposed method is based on the U.S.P. colorimetric epinephrine assay (2) which is adapted from a paper by Doty (3). In this method, epinephrine in a solution suitably buffered about pH 8.5 forms with ferrous ion a color having maximum absorbance at 530 mμ. Doty reports that the color is given by all catechols tested but not by phenols or by dihydroxy phenols in which the phenolic groups are not *ortho* to each other. The reaction is stoichiometric, 0.5 mole of ferrous ion being required per mole of catechol.

The U.S.P. requires about 200 mcg. of epinephrine in the sample. The color developed is compared with color developed in a similar manner with U.S.P. epinephrine bitartrate reference standard. By using a suitable standard, acceptable accuracy may be obtained with a sample as small as 50 mcg. When epinephrine is used as a vasoconstrictor in preparations for dental anesthesia, the recommended concentrations are 1:50,000 (20 mcg./ml.) or 1:100,000 (10 mcg./ml.). As high as 1:20,000 (50 mcg./ml.) are found in commercial preparations. Only at the highest concentration is the colorimetric method, as specified in the U.S.P., satisfactory for a 1-ml. sample.

METHOD

Reagents.—Epinephrine bitartrate reference solution, dissolve 18.2 mg. of U.S.P. epinephrine bitartrate reference standard plus 200 mg. $NaHSO_3$ in enough water to make 50.0 ml. Epinephrine working standard, dilute 5.00 ml. of epinephrine bitartrate reference solution with enough water to make 50.0 ml. (1:50,000). Buffer stock solution, dissolve 42 Gm. of $NaHCO_3$ and 50 Gm. of $KHCO_3$ in about 180 ml. of water, dissolve 37.5 Gm. of aminoacetic acid and 17 ml. of strong ammonia solution, U.S.P. (28% NH_3) in about 180 ml. of water, mix the solutions, and dilute to 500 ml. Buffer solution, pH 9.0, dilute 10 ml. of the buffer stock solution to 100 ml. Standard ferrous salt solution, dissolve 47.8 mg. of ferrous ethylenediamine sulfate $FeC_2H_4(NH_3)_2(SO_4)_2 \cdot 4H_2O$, standard of reference grade (G. Frederick Smith Chemical Co., 1867 McKinley Ave., Columbus, Ohio), 5 ml. 0.1 *N* hydrochloric acid, and 0.5 Gm. $NaHSO_3$ in water to make 100.0 ml. Each ml. contains 1.25 μm. of ferrous ion equivalent to 2.50 μm. of catechol.

Equipment.—Gilmont micropipet-buret, 0.1-ml. size (Manostat Corp., 20 N. Moore St., New York 3, N. Y.). Beckman B spectrophotometer with 1/4 in. wooden blocks in cell compartments to raise cells in light path and reduce required volume.

Procedure.—Pipet into the reference cell 1.00 ml. water, 1.0 ml. of buffer solution, and 0.5 ml. of diluent, and mix. Pipet into the sample cell 1.00 ml. of sample, 1.0 ml. of buffer solution, and 0.5 ml. of diluent, and mix. With the wavelength setting at 530 mμ and a sensitivity of 1, the slit width required for balancing the instrument with the reference solution in the light path is about 0.18 mm. Record the absorbance of the sample solution. Add at least three aliquots of standard ferrous salt solutions so that the total added is less than 50% of the volume calculated to be required at the end point, with no aliquot less than 0.005 ml., recording the absorbance of the sample after each addition. Add enough standard ferrous salt solution so that the total added is 1.5 times the amount calculated to be required by the sample, record the absorbance of the sample, then add at least two additional 0.01-ml. aliquots, recording the absorbance after each addition. Plot total volumes added *vs.* absorbance. The first set of points will define a line of positive slope; the second set, a horizontal line. The intersection of the two lines defines the end point (4). Calculate the amount of catechol present in the sample. A complete discussion of photometric titration, including typical curves, is given by Goddu and Hume (4).

Assays of commercial lots were run in the same manner using isopropyl alcohol as the diluent to keep the anesthetic bases in solution.

Results of assays are given in Tables I and II.

DISCUSSION

The maximum observed absorbance for 1:100,000 solutions was about 0.040 with 0.005-ml. increments of titrant producing increments in absorbance of about 0.007. The maximum observed absorbance for 1:50,000 solutions was about 0.080 with 0.01-ml. increments of titrant producing increments of absorbance of about 0.015. The maximum observed absorbance for 1:20,000 solution was about 0.190 with 0.02-ml. increments of titrant producing increments in absorbance of about 0.040. Despite the low photometric accuracy in each individual observation that might be expected at these low ab-

Received June 14, 1961, from the Department of Chemistry, School of Pharmacy, Temple University, Philadelphia 40, Pa.

Accepted for publication October 5, 1961.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

TABLE I.—ASSAY OF ALIQUOTS OF REFERENCE SOLUTION

Series A. Water diluent	Epinephrine, mcg. Theory	Found	Recovery, %	
1	20.0	19.3	97	
2		19.4	97	
3		19.3	97	
4		19.0	95	
				Av. 19.2 mcg.
				S.D. 0.2 mcg.
Series B. Isopropyl alcohol diluent				
5		19.1	95	
6		20.4	102	
7		20.3	101	
				Av. 19.8 mcg.
				S.D. 0.8 mcg.
Series C. Acetone diluent				
8		19.7	99	
9		20.6	103	
				Av. 20.1 mcg.
				S.D. 0.7 mcg.

Average of 9 determinations 19.7 mcg., S.D. 0.5 mcg.

TABLE II.—ASSAY OF COMMERCIAL LOTS

Lot	Anesthetic Present	Claimed, mcg.	Found, mcg.	
A	2% Procaine · HCl (epinephrine 1:50,000)	20.0	22.2	
			22.0	
			22.4	
				Av. 22.2 mcg.
B	2% Lidocaine · HCl (epinephrine 1:100,000)	10.0	12.5	
			12.7	
			12.3	
			12.6	
				Av. 12.5 mcg.
				S.D. 0.2 mcg.
C	2% Lidocaine · HCl (epinephrine 1:100,000)	10.0	11.8	
			11.8	
			11.5	
				Av. 11.7 mcg.
				S.D. 0.2 mcg.
D[a]	2% Mepivacaine · HCl (epinephrine 1:20,000)	50.0	49.0	
			46.9	
			47.6	
				Av. 47.8 mcg.
				S.D. 1.3 mcg.

[a] 1-Nordefrin, an isomer of epinephrine, present as vasoconstrictor.

sorbance readings, the relative standard deviation of the results did not exceed 5% in any determination and was usually about 2%. It may be concluded, therefore, that the reproducibility of the assay using 10 mcg. of epinephrine in the sample is of about the same order as the U.S.P. photometric method using 200 mcg. of epinephrine in the sample.

The actual titration requires about 10 minutes. If preparation of solutions and calculations of results are included, three samples may be completed in an hour.

The method allows the smaller laboratory to assay all catechols without the necessity of maintaining a reference standard for each. The single ferrous salt standard is all that is required. The primary standard grade of ferrous ethylenediamine sulfate is stable and has an infinite shelf life. Both in stability and cost it is to be preferred to a catecholamine reference compound.

The proposed method also has served as a check on the U.S.P. epinephrine bitartrate reference standard. By comparison with the standard ferrous salt, the reference standard is not less than 99.5% pure.

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Identification and Purity Test for Ergonovine and Methylergonovine

By THOMAS G. ALEXANDER

A method is described for the separation and identification of those "water-soluble" ergot alkaloids of pharmaceutical interest; namely, ergonovine, ergonovinine, methylergonovine, and methylergonovinine.

THE IDENTIFICATION tests in the official compendia (1, 2) fail to distinguish between ergonovine (the principal oxytocic agent of ergot), methylergonovine (a synthetic homolog), and the dextrorotatory, physiologically inactive isomers, ergonovinine and methylergonovinine. This study was undertaken for the purpose of developing a suitable test for the identification of ergonovine and methylergonovine in pharmaceuticals, and for the detection of possible contaminants and breakdown products. In searching for such a test, several of the paper chromatographic procedures reported in the literature were investigated. With each of the partitioning procedures described by Foster, *et al.* (3), Macek (4), Voigt and Weiss (5), and Alexander and Banes (6) we could effectively separate the dextrorotatory alkaloids from their diastereoisomers. Attempts to separate ergonovine and methylergonovine from each other by these and similar procedures were unsuccessful. The reverse-phase paper chromatographic procedure described by Stoll and Rüegger (7) included an overnight equilibration of the spotted paper in the vapors of the mobile phase. It was our experience that, with this procedure, each of the isomers would partially convert to its diastereoisomer. Pöhm and Fuchs (8) and Horak and Kudrnac (9) described methods incorporating benzene into the mobile solvent. In the hands of the author, these ergot alkaloids decomposed in the presence of benzene.

Berg (10) described a method for the separation of water-soluble ergot alkaloids as well as the products of hydrolysis, ergine, and lysergic acid. Circular wick paper chromatographic technique was used. Papers were impregnated with pH 6.4 buffer and chromatograms developed with water-saturated methylene chloride. The method described below is an adaptation of Berg's system incorporating the simpler ascending paper chromatographic techniques described by Mitchell (11) and the "moist-paper" technique described by Levine and Fischbach (12).

MATERIALS AND METHOD

Mobile Solvent.—Mix 50 ml. of ethylene chloride, 50 ml. of *n*-butyl acetate, 7 ml. of pyridine, and 2 ml. of water in a separator. Allow to settle, draw off the bottom layer through a glass wool filter, and discard the top layer.

Standard Solutions.—Dissolve 10 mg. of methylergonovine maleate N.F. XI, or ergonovine maleate U.S.P. XVI in 2.0 ml. of alcohol. This is *standard solution A*. Dilute 1.0 ml. of standard solution A to 25 ml. with alcohol to obtain *standard solution B*.

Sample Solution.—Dissolve ergonovine maleate or methylergonovine maleate powder in a suitable quantity of alcohol to obtain a solution strength of 5 mcg. per μl. To analyze an injection solution, render a suitable portion alkaline with sodium bicarbonate, extract with chloroform, evaporate the extract to dryness, and dissolve the residue in a quantity of alcohol sufficient to obtain a solution strength equivalent to 5 mcg. ergonovine maleate or methylergonovine maleate per μl. To analyze tablets, prepare chloroform extracts as described under *Identification* in the N.F. monograph for methylergonovine maleate tablets (2). Dissolve the dried residues in a quantity of alcohol sufficient to obtain a solution strength equivalent to 5 mcg. per μl.

Procedure.—Arrange a chromatographic tank suitable for ascending paper chromatography. Add mobile solvent, seal the tank, and equilibrate for 1 hour. Pour about 10 ml. aqueous buffer solution pH 4.6 [such as U.S.P. phthalate buffer (13)] over premarked paper. After draining for 30 seconds, place the paper between sheets of paper toweling. Blot the surface with the toweling for some seconds to remove as much moisture as will be absorbed, and repeat using fresh towels. Spot 0.010 ml. of each of the standard solutions and volumes of sample solutions equivalent to 50 mcg. ergonovine maleate or methylergonovine maleate. Rewet the spots with water from a capillary tube. In addition, spot methyl red U.S.P. XVI, T.S., at the edge of the paper to aid in following the solvent front. The paper should feel definitely moist and limp, but not so wet as to transfer water to the fingers. Place the paper in the chromatographic tank and reseal.

When the solvent front is within 2 cm. of the top of the paper (about 1 hour), remove it and dry in a hood. Examination under ultraviolet light should show one primary spot with an R_f and an intensity corresponding to that of the respective standard A spot. Any other spot occurring on the sample chromatogram represents impurities. The standard B (2 mcg.) spot is used to estimate roughly the quantity of any such impurities.

The lysergic acid moiety of these ergot alkaloids will react with strongly acidic *p*-dimethylaminobenzaldehyde to give a blue color. By spraying the developed chromatogram with an alcoholic, acidic solution of this aldehyde, as described by Hellburg (14), and photographing the dried chromatogram, permanent records may be obtained.

RESULTS AND DISCUSSION

Typical R_f values obtained are 0.19 for ergonovine, 0.35 for methylergonovine, 0.44 for ergonovinine, and 0.60 for methylergonovinine. Any water-insoluble ergot alkaloids present as contaminants will be indicated by spots located at the solvent front. Ergonovinine and methylergonovinine used in the study were prepared by the method described by Kleiderer (15) whereby 5 mg. of the levorotatory isomer is dissolved in 1 ml. of methyl alcohol, two drops of concentrated ammonia added, and the solution kept at room temperature overnight.

Received July 19, 1961, from the Department of Health, Education, and Welfare, Food and Drug Administration, Division of Pharmaceutical Chemistry, Washington 25, D. C.
Accepted for publication August 14, 1961.

About half of the alkaloids are converted to the dextrorotatory form by this procedure.

When attempting to adapt Berg's system to a simpler apparatus, ergonovine and methylergonovine were well separated; but spots were very diffuse and the developing solvent failed to ascend the paper evenly. More satisfactory chromatograms were obtained by diluting the developing solvents with *n*-butyl acetate. Less volatile chlorinated solvents would not separate ergonovine and methylergonovine. Of the volatile chlorinated solvents, only methylene chloride or ethylene chloride would separate the homologs and then only when they constituted half, or more, of the developing solvent.

By simultaneously increasing the acidity of the impregnating solution and the basicity of the mobile solvent as compared to conditions prescribed by Berg, less diffuse spots were obtained while maintaining about the same R_f values. In analyzing alkaloid samples by the procedure described above, unsatisfactory chromatograms were occasionally obtained where the alkaloids had migrated excessively or insufficiently during development. The mobility of the alkaloids could be increased by raising the pH of the mobile solvent or decreased by lowering the pH. The R_f values reported are typical of those obtained. When desired for special cases, greater than usual separation of ergonovine and methylergonovine, or greater separation of a pair of diastereoisomers, can be achieved by such a pH adjustment.

Several techniques were tried for conditioning the paper prior to development. Among these is the commonly used technique whereby papers are impregnated, dried, spotted, moistened by equilibration, and developed. Most satisfactory results were obtained by the "moist-paper" technique described above. Grossly diffuse spots are obtained if the papers are too wet when placed in the tank and immobility of the spots will result if the paper is too dry.

A methylergonovine maleate tablet mix and several injection solutions, of both ergonovine maleate and methylergonovine maleate, were prepared to simulate market products. These were then analyzed by the procedure described in this paper. The results indicated that there is no deterioration of alkaloid during analysis and that the excipients do not interfere. The procedure described in this paper was successfully applied to 14 commercial samples. Neither of the four samples of alkaloid salts, nor any of the seven tablet samples analyzed, contained more than trace quantities (about 1%) of the respective diastereoisomer. Only one of three injection solutions tested contained any dextrorotatory isomer and that, only after a year of storage under refrigeration. The chromatogram for another one of these injection solutions exhibited faint spots representing material not containing the lysergic acid moiety. These may have represented oxidized alkaloids.

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$$\frac{d \ln S}{dT} = \frac{\Delta H}{RT^2}$$

which when integrated becomes

$$\log S = \frac{-\Delta H}{2.303\,RT} + C$$

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About half of the alkaloids are converted to the dextrorotatory form by this procedure.

When attempting to adapt Berg's system to a simpler apparatus, ergonovine and methylergonovine were well separated; but spots were very diffuse and the developing solvent failed to ascend the paper evenly. More satisfactory chromatograms were obtained by diluting the developing solvents with *n*-butyl acetate. Less volatile chlorinated solvents would not separate ergonovine and methylergonovine. Of the volatile chlorinated solvents, only methylene chloride or ethylene chloride would separate the homologs and then only when they constituted half, or more, of the developing solvent.

By simultaneously increasing the acidity of the impregnating solution and the basicity of the mobile solvent as compared to conditions prescribed by Berg, less diffuse spots were obtained while maintaining about the same R_f values. In analyzing alkaloid samples by the procedure described above, unsatisfactory chromatograms were occasionally obtained where the alkaloids had migrated excessively or insufficiently during development. The mobility of the alkaloids could be increased by raising the pH of the mobile solvent or decreased by lowering the pH. The R_f values reported are typical of those obtained. When desired for special cases, greater than usual separation of ergonovine and methylergonovine, or greater separation of a pair of diastereoisomers, can be achieved by such a pH adjustment.

Several techniques were tried for conditioning the paper prior to development. Among these is the commonly used technique whereby papers are impregnated, dried, spotted, moistened by equilibration, and developed. Most satisfactory results were obtained by the "moist-paper" technique described above. Grossly diffuse spots are obtained if the papers are too wet when placed in the tank and immobility of the spots will result if the paper is too dry.

A methylergonovine maleate tablet mix and several injection solutions, of both ergonovine maleate and methylergonovine maleate, were prepared to simulate market products. These were then analyzed by the procedure described in this paper. The results indicated that there is no deterioration of alkaloid during analysis and that the excipients do not interfere. The procedure described in this paper was successfully applied to 14 commercial samples. Neither of the four samples of alkaloid salts, nor any of the seven tablet samples analyzed, contained more than trace quantities (about 1%) of the respective diastereoisomer. Only one of three injection solutions tested contained any dextrorotatory isomer and that, only after a year of storage under refrigeration. The chromatogram for another one of these injection solutions exhibited faint spots representing material not containing the lysergic acid moiety. These may have represented oxidized alkaloids.

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Correlation Between Solubility Parameters and Dielectric Constants

Sir:

There is general agreement that one of the fundamental problems in pharmaceutical research involves the description of solubility phenomena. In attempting to explain these phenomena we usually resort to either qualitative description in terms of relative "polarity" or more quantitatively with the aid of the thermodynamic approach embodied in Hildebrand's solubility parameter concept (1). It is the purpose of this communication to present some of our observations relevant to these two approaches.

Some earlier solubility studies of pharmaceutical importance (2–4) have illustrated the existence of a peak solubility of solutes when the components of cosolvent mixtures were varied in concentration. In these cases, the treatment of solubility offers no quantitative explanation for this behavior.

Moore (5), however, suggested a semiempirical use of dielectric constants to solvent mixtures to predict the acceptability of cosolvent blends to maintain solubility. Although the peak solubility or dielectric requirement had not been determined, this work illustrates the usefulness of employing an appropriate dielectric constant in the choice of a solvent or solvent mixture for the maintenance of solubility for a given solute. A more comprehensive study reported by Semenchenko (6) shows that the solubility of some 41 solutes have been measured as a function of the dielectric constant of the solvent system and claims that "... an ascending branch and descending branch are observed, including a peak value for the solubility ...." In this case, a distribution curve is described wherein the peak value is dependent on the characteristics of both the solute and solvent system and can be considered the dielectric requirement of the solute.

On the other hand, Chertkoff and Martin (7) recently applied the solubility parameter concept (1) in their studies of the solubility of benzoic acid in mixed solvent systems. This was an attempt to extend Hildebrand's theory of solubility to "semipolar" solutes. They, too, find a peak solubility as a function of solubility parameters of the solvent system in accord with the theoretical curve predicted by Hildebrand's equation.

In treating solubility data, whether in terms of dielectric constants or solubility parameters, the end results are similar. This immediately suggests that at least an empirical relationship exists between the two concepts. The following paragraphs present further observations to substantiate this contention and show the utility of such a relationship.

The first example is illustrated in Fig. 1 which shows a plot of both solubility parameters and dielectric constants *vs.* the number of carbon atoms for a homologous series of *n*-alkyl alcohols (8). When the solubility parameter and dielectric constant of one member is matched

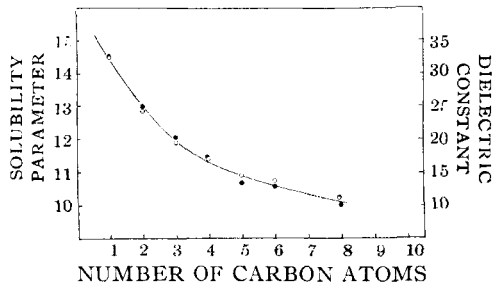


Fig. 1.—An illustration of the superimposition of curves when either the solubility parameter, O, or dielectric constant, ●, of a series of *n*-alkyl alcohols is plotted *vs.* the number of carbon atoms.

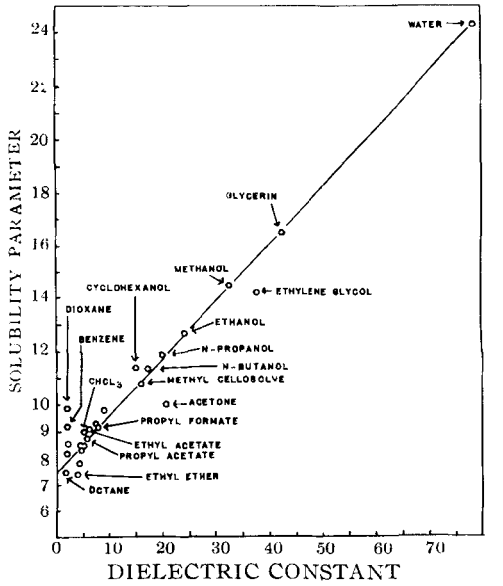


Fig. 2.—A plot of solubility parameters of common solvents *vs.* their respective dielectric constants to show the linear trend.

on a sliding ordinate, the two curves become superimposable. Obviously, a plot of solubility parameters *vs.* dielectric constants for these alcohols would result in a straight line.

Furthermore, by extending representative solvents beyond mere alcohols to include various classes of solvents, a similar straight line plot of solubility parameter *vs.* dielectric constant should also result. This was essentially found to be the case with 25 solvents and is illustrated in Fig. 2. The equation for this straight line can be written

$$\delta = 0.22\,\epsilon + 7.5 \qquad \text{(Eq. 1)}$$

where δ is the solubility parameter and ϵ is the dielectric constant. The intercept value is obtained in the extrapolation to $\epsilon = 1$.

To show the utility of this equation, we have analyzed the data on the solubility of phenobarbital from several sources. The results are summarized in Table I. It can be seen that irrespective of the composition of solvent mixtures showing peak solubility for phenobarbital, there are striking similarities when their dielectric constants are compared.

Similarly, using data reported by Chertkoff and Martin (7) one can calculate the dielectric requirement of benzoic acid. Equation 1 predicts a value of 18.2 from the reported solubility parameter of 11.5 for benzoic acid. Analysis of solubility data given by Seidell (9) predicts a dielectric requirement of about 20 for benzoic acid. These examples are given to illustrate how completely independent sources of data aid to confirm the validity of Eq. 1.

There are some obvious advantages to the correlation of solubility parameters with dielectric constants. For example, the range of solubility parameters is quite small with values falling between 7.5 for "nonpolar" compounds, and about 24.3 for water. The dielectric constant spectrum, on the other hand, varies from about 2 to 80, giving over a fivefold expansion of a reference scale from that of solubility parameters and would follow a regular order with respect to "polarity."

TABLE I.—SUMMARY OF PROPERTIES OF SOLVENTS SHOWING PEAK SOLUBILITY FOR PHENOBARBITAL

Composition, v/v	Dielectric Constant[a]	Solubility Parameter[b]	Ref.
Ethanol-water (9:1)	26.6	13.23	2
Ethanol-glycerin (8:2)	25.7	13.15	2
Ethanol-propylene glycol (1:1)	26.5	13.23	3

[a] Experimentally measured by the resonance method. [b] Determined from Eq. 1.

Secondly, dielectric constants of solvents are more easily determined experimentally than solubility parameters. Furthermore, the solubility parameters of solids can ordinarily be obtained only after elaborate approximations of physical data are made. Thus, it appears that measurement of dielectric constants provides convenient means for determining apparent solubility parameters through Eq. 1, with all the incumbent advantages for applications of such knowledge.

It is obvious from Fig. 2 that, in general, the solvents which associate primarily through hydrogen bonding give the best correlation between reported solubility parameters and their respective dielectric constants. Fortuitously, these are the solvents of pharmaceutical importance.

Greatest deviation from the linear trend in Fig. 2 occurs with the nonpolar solvents which are of less pharmaceutical significance. The values for these solvents all fall in clusters in a small segment in the lower portion of the spectrum of dielectric constants. This scatter may be due, in part, to the diversity of chemical types and the slight variations in the reported values of solubility parameters, especially when different methods were employed for their determination.

It would be of interest to determine under what circumstances the solubility parameter concept might be extended into the semipolar and polar solvents and to determine if the empirical Eq. 1 has any physical significance. Each is currently being investigated in these laboratories and our findings shall be reported.

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Received March 23, 1962.
Accepted for publication May 31, 1962.

Gasket Insert Modification of U.S.P. Tablet Disintegration Apparatus

Sir:

The U.S.P. XVI apparatus for tablet disintegration testing specifies a basket rack assembly consisting of six open glass tubes each 7.75 cm. long and having an inside diameter of approximately 21.5 mm. Each tube is provided with a slotted and perforated cylindrical disk 9.5 mm. thick and 20.7 mm. in diameter.

The procedure directed for disintegrating uncoated and plain coated tablets is to place one tablet in each of the six tubes of the basket, add a disk to each tube, and then operate the apparatus as indicated in the tablet disintegration test monograph.

It has been our experience that many tablets will lie flat on the bottom wire screen, or because of gummy and gelatinous ingredients or ingredients of an adhesive character, either will adhere to the rising and falling disks, or will adhere to the bottom stainless steel wire cloth. In any case, the tablet disintegration system intended by the U.S.P. does not apply. Instead, the net result may be a flattening out of the tablet core caused by the constant action of the falling disk on the same point of the tablet surface. With other tablets, an indeterminate soft mass results which makes exact timing of the disintegration end point difficult.

We have found that by inserting a thin, flexible rubber gasket in the bottom of the glass tubes in contact with the lower stainless steel wire cloth, an increased turbulence is effected in the immersion fluid as the basket rack assembly rises and falls. This turbulence causes the tablets to "bob" up and down, increases the rate of flow of fluid around the tablets, creates more uniform attrition to all of the tablet surfaces, and prevents adhesion of the tablets to the bottom wire cloth. Thus it has served to obviate the use of the disks in tablet disintegration testing in most cases.

As an additional application, the introduction of these gasket inserts permits the use of the U.S.P. disintegration apparatus for establishing *in vitro* disintegration patterns for timed-release and sustained-action tablets. The rate of flow of the fluids around the tablets simulates the flow rate realized in the rotating bottle method (1) recommended by Krueger and Vliet to the P.M.A. Contact Section (2).

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Received March 27, 1962.
Accepted for publication April 11, 1962.

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Book Notices

Los Colorantes Sinteticos en Bromatologia y Farmacia. By J. M. PLA DELFINA. Sociedad Española de Farmacotecnia, Barcelona, Spain, 1961. 372 pp. 16 × 23 cm. Paperbound. Price 200 pesetas.

This monograph (Spanish) includes a brief summary of the legal status of colors used in different countries; it classifies the various colors and describes properties, uses, and methods for detection and determination of coloring materials.

Organic Syntheses. Vol. 41. Edited by JOHN D. ROBERTS. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1961. ix + 118 pp. 15 × 23 cm. Price $4.

This volume includes methods for 28 compounds. The smallest-scale synthesis yet given is for 0.0005 mole of cholestanyl methyl ether. More than a third of the reactions and compounds described in this volume were unknown five years ago. One illustrative procedure, for the extraordinary conversion of carboxylic acids to trifluoromethyl groups by sulfur tetrafluoride, is included within 18 months after its original publication.

Textbook of Organic Medicinal and Pharmaceutical Chemistry. 4th ed. Edited by CHARLES O. WILSON and OLE GISVOLD. J. B. Lippincott Co., Medical Dept., East Washington Square, Philadelphia 5, Pa., 1962. xvi × 883 pp. 18 × 25.5 cm. Price $14.

This established textbook utilizes chemical and pharmacologic classifications to present its text material that has been revised to include the latest available medicinal compounds and other materials of pharmaceutical importance. A Chapter on Physicochemical properties in relation to biologic action has been included. Products in U.S.P. XVI, N.F. XI, N.N.D. 1961, and A.D.R. 1961 have been covered in this edition.

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

August 1962 volume 51, number 8

Review Article

Current Status of Research on the Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don)

By GORDON H. SVOBODA, IRVING S. JOHNSON, MARVIN GORMAN, and NORBERT NEUSS

THE PANTROPICAL apocynaceous plant *Vinca rosea* Linn. is an erect, everblooming pubescent herb or subshrub, 1 to 2 ft. high. It is cosmopolitan in the tropics and is widely cultivated in gardens throughout the world as an ornamental. The pink and the white color varieties are found in the natural state, while three seed hybrids, *viz.*, blush pink with red eye, crimson, and white with red eye, are also commerically available.

Generally accepted synonyms for the genus *Vinca* are *Pervinca*, *Lochnera*, and *Catharanthus*. Pichon (2) has shown that the genera *Vinca* and *Lochnera* differ in 34 morphological characteristics and should not be used as synonyms. He furthermore found that *Catharanthus* G. Don predated *Lochnera* Reichb. by 7 months. While it would appear that the correct botanical name for this plant is *Catharanthus roseus* G. Don, we shall continue to use the more common *Vinca rosea* Linn. synonymously.

An early description of the medicinal properties of this plant mentions that an infusion of the leaves was used in Brazil in the treatment of hemorrhage and scurvy, as a mouthwash for toothache, and for healing and cleaning chronic wounds (3). Recently it has been reported that the total alkaloids possess a limited antibiotic activity against *V. cholera* and *M. pyogenes* var. *aureus*, as well as a significant and sustained hypotensive action (4). A recent review by Farnsworth (5) contains an exhaustive summary of early descriptions of medicinal properties of the plant, as well as those of other members of these genera.

The folklore reputation which this plant enjoyed as an oral hypoglycemic agent (6) prompted its phytochemical examination in two different laboratories, independently and unknown to each other. While neither group could substantiate this reported activity in either normal or experimentally-induced hyperglycemic rabbits, the Canadian group of Noble, Beer, and Cutts observed a peripheral granulocytopenia and bone marrow depression in rats associated with certain fractions (7, 8). Continued investigation led to their preparation of vincaleukoblastine (VLB) sulfate,[1] an alkaloid capable of producing severe leukopenia in rats (9–11). The Lilly group had demonstrated that certain alkaloidal fractions gave a reproducible effectiveness in DBA/2 mice infected with the P-1534 leukemia, a transplanted acute lymphocytic leukemia (12–15). An intensive phytochemical examination resulted in obtaining leurosine,[1] a new dimeric alkaloid closely chemically related to VLB, as well as VLB sulfate. The effectiveness of both of these alkaloids against the P-1534 leukemia in DBA/2 mice was first demonstrated in these laboratories.

Received from the Organic Chemical Development and Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Paper XIII (a review) in the series; the preceding paper appeared in THIS JOURNAL (1).

[1] The A.M.A. Council on Drugs has approved vinblastine, vinleurosine, vinrosidine, and vincristine as generic names for these alkaloids. VLB is supplied as Velban (vinblastine sulfate, Lilly).

Our early recognition of the fact that the "indefinite" survivors observed with certain amorphous fractions were due neither to VLB nor leurosine, nor to their combination, further stimulated the phytochemical search for the compound(s) responsible for this profound activity. These efforts culminated in the discovery of leurosidine[1] and leurocristine (16).[1]

PREPARATION OF ALKALOIDS

The earliest chemical investigation of this plant was conducted by Greshoff (17) who reported the presence of alkaloids, but who was unable to obtain pure crystalline compounds. Cowley and Bennett (18) prepared a crystalline tartrate and two sulfates from the total alkaloidal extract, but did not describe their physical or chemical properties. More recently, several groups of investigators have obtained the following alkaloids: ajmalicine (19), akuammine (19), tetrahydroalstonine (20), serpentine (19, 20), lochnerine (21), and reserpine (22), as listed in Table I. Inasmuch as these alkaloids are well documented, they do not merit further discussion.

During the course of our investigation, Beer obtained VLB as the sulfate (9), the preparation of which we were able to duplicate. Kamat and co-workers (23) reported on two amorphous and two crystalline alkaloids, one of which they described in detail and named vindoline, apparently being identical to our vindoline (24). An alkaloid, independently named lochnericine, has been reported by Nair and Pillay (25), and appears to be identical with our lochnericine (24). The occurrence of vindolinine was established simultaneously by Janot and co-workers (26) and our group, and the name was selected by mutual agreement.

The alkaloids reported to date solely from these laboratories include: leurosine, virosine, perivine, catharanthine, isoleurosine, lochneridine, sitsirikine, vincamicine, catharine, vindolicine (27), leurosidine, leurocristine (16), carosidine, carosine, pleurosine, neoleurosidine, vincarodine, catharicine, vindolidine, and neoleurocristine (1). These alkaloids, along with VLB and the three co-discovered ones, vindoline, lochnericine and vindolinine, are listed in Table II, along with their empirical formulas, m.p.'s, pK'a's, specific rotations, and ultraviolet absorption maxima.

TABLE I.—ALKALOIDS FROM *Vinca rosea* LINN.

Name	Formula
Ajmalicine	$C_{21}H_{24}N_2O_3$
Tetrahydroalstonine	$C_{21}H_{24}N_2O_3$
Serpentine	$C_{21}H_{22}N_2O_3$
Lochnerine	$C_{20}H_{24}N_2O_2$
Akuammine[a]	$C_{22}H_{26}N_2O_4$
Reserpine[a]	$C_{33}H_{40}N_2O_9$

[a] Akuammine (vincamajoridine) and reserpine have not been encountered in our investigation, while the presence of the other four has been confirmed.

Figure 1 represents the scheme used in the extraction of the drug. The process consists essentially of separating the plant alkaloids into those whose tartrates are soluble in organic solvents and those which are not, under the existing conditions. The basis for this method, in which the tartaric acid serves to bind the stronger bases in the crude drug, affording an initial separation of the weak and strong bases during extraction, was first described in 1957 (28) in a paper dealing with the root bark alkaloids of *Alstonia constricta* F. Muell.

The final purification of most of the alkaloids listed in Table II was achieved by elution chromatography on alumina deactivated by treatment with 10% acetic acid. The eluting solvents used were benzene, benzene-chloroform mixtures, chloroform, and chloroform-methanol mixtures. In order to obtain a number of the alkaloids it was necessary to subject certain selected post-VLB column fractions to a gradient pH technique, which is described as follows: A 10-Gm. sample of the crude amorphous chromatographic fraction was dissolved in 500 ml. of benzene. Any benzene-insoluble material which would result was removed by filtration and discarded. The alkaloids were extracted into 500 ml. of 0.1 *M* citric acid by a reduced pressure steam distillation. Insoluble material was removed by filtration and discarded. The filtrate volume was then adjusted to 500 ml. with water. The resulting clear acidic solution was extracted at pH levels of 2.80, 3.40, 3.90, 4.40, 4.90, 5.40, 5.90, 6.40, and 7.50 (± 0.02), with 500-ml. portions of benzene. The pH of the aqueous phase was raised by the addition of ammonia. The benzene extracts were dried over sodium sulfate and were concentrated *in vacuo* to dryness. The alkaloids were crystallized therefrom by the addition of either methanol or acetone.

It must be stated that no one column operation yielded every alkaloid that is listed as being obtained from a particular starting fraction. The following schemes, as presented in Figs. 2 and 3, will serve to illustrate the manner in which all of these alkaloids were obtained.

BIOLOGICAL PROPERTIES

Four of the recently isolated alkaloids from *Vinca rosea* Linn. have been of particular biological interest in these laboratories because of

TABLE II.—NEW ALKALOIDS FROM *Vinca rosea* LINN.

Name	Formula	M.p., °C.[a]	$[\alpha]_D^{26}$ $CHCl_3$	pK'a in DMF	U.V. $\lambda_{max.}^{EtOH}$, mμ[a]
Leurosine	$C_{46}H_{58}N_4O_9$[b]	202–205 (decompn.)	+72	5.5, 7.5 (H_2O)	214, 259
Vincaleukoblastine	$C_{46}H_{56}N_4O_9$	211–216 (decompn.)	+42[c]	5.4, 7.4 (H_2O)	214, 259
Virosine	$C_{22}H_{26}N_2O_4$[b]	258–264 (decompn.)	−160.5	5.85 (66%)	226, 270
Perivine	$C_{20}H_{24}N_2O_3$	180–181	−121.4	7.5 (66%)	226, 314
Catharanthine	$C_{21}H_{24}N_2O_2$	126–128	+29.8	6.8 (66%)	226, 284, 292
Lochnericine	$C_{21}H_{24}N_2O_2$	190–193 (decompn.)	−432	4.2 (66%)	226, 297, 327
Vindolinine·2HCl	$C_{21}H_{24}N_2O_2 \cdot 2HCl$	210–212 (decompn.)	−8 (H_2O)	3.3, 7.1 (66%)	245, 300
Vindoline	$C_{25}H_{32}N_2O_6$	154–155	−18	5.5 (66%)	212, 250, 304
Isoleurosine	$C_{46}H_{60}N_4O_9$[b]	202–206 (decompn.)	+61.2	4.8, 7.3 (66%)	214, 261, 287
Lochneridine	$C_{20}H_{24}N_2O_3$[d]	211–214 (decompn.)	+607.5	5.5 (66%)	230, 293, 328
Sitsirikine·1/2 H_2SO_4	$C_{21}H_{26}N_2O_3 \cdot 1/2$ H_2SO_4	239–241 (decompn.)	+23 (Base)	7.6 (66%)	224, 282, 288
Vincamicine	(Dimeric)	224–228 (decompn.)	+418	4.80, 5.85 (66%)	214, 264, 315, 341
Catharine	$C_{46}H_{52}N_4O_9 \cdot CH_3OH$	271–275 (decompn.)	−54.2	5.34 (66%)	222, 265, 292
Vindolicine	$(C_{25}H_{32}N_2O_{62})$	248–251 (melts, recryst.) 265–267 (decompn.)	−48.4	5.4 (66%)	212, 257, 308
Leurosidine	(Dimeric)	208–211 (decompn.)	+55.8	5.0, 8.8 (33%)	214, 265
Leurocristine	$C_{46}H_{54}N_4O_{10}$	218–220 (decompn.)	+17.0 ($EtCl_2$)	5.0, 7.4 (33%)	220, 255, 296
Carosidine	(Dimeric)	263–278, 283 (decompn.)	−89.8	...	212, 254, 303
Carosine	$C_{46}H_{56}N_4O_{10}$[b]	214–218	+6.0	4.4, 5.5 (33%)	255, 294
Pleurosine	$C_{46}H_{56}N_4O_{10}$[b]	191–194 (decompn.)	+61.0	4.4, 5.55 (33%)	267, 308
Neoleurosidine	$C_{48}H_{62}N_4O_{11}$[b]	219–225 (decompn.)	+41.6	5.1 (33%)	214, 268
Vincarodine	$C_{44}H_{52}N_4O_{10}$[b]	253–256 (decompn.)	−197.4	5.8 (66%)	230, 272, 298
Catharicine	$C_{46}H_{52}N_4O_{10}$[b]	231–234 (decompn.)	+34.8	5.3, 6.3 (33%)	214, 268, 293, 315
Vindolidine	$C_{48}H_{64}N_4O_{10}$[b]	244–250 (decompn.)	−113.2	5.3 (33%)	261, 311
Neoleurocristine	$C_{46}H_{56}N_4O_{12}$[b]	188–196 (decompn.)	−57.87	4.68 (33%)	220, 257, 298

[a] The melting points were determined on a Kofler microstage. The ultraviolet absorption spectra were obtained using a Cary model 14 spectrophotometer. [b] While these molecular formulas agree well with the analytical results for each particular alkaloid, it should be noted that they are to be considered as proximate at this time, in light of our experience with the other dimeric alkaloids (58). [c] Determined on VLB etherate. [d] On the basis of mass spectrometric evidence obtained by H. Budzikiewicz and J. M. Wilson of Stanford, we now prefer the C_{20} formulation rather than the C_{19} first reported (27).

their effects as chemotherapeutic agents in experimental neoplasias. These are: vincaleukoblastine, leurosine, leurocristine, and leurosidine. The transplantable tumor used as a bioassay for these alkaloids is the P-1534 leukemia. This is an acute lymphocytic leukemia which, in our laboratory, is transplanted only in DBA/2 mice. It has been used to detect and predict clinical activity of a number of antineoplastic compounds (13, 15, 29–34).

The techniques by which drugs are assayed with this and other tumors in our laboratory have been previously described (15).

The activity observed with the initial total extract of the plant and with the first crude fractions is shown in Table III. This led to the production of leurosine and VLB. Their activity against the P-1534 leukemia and other murine tumors has been described in detail (15, 34). Briefly, animals implanted with the P-1534 leukemia and treated with VLB survived 40–150% longer than saline-treated controls, when VLB was administered at levels ranging from 0.05–0.6 mg./Kg. Similar results were obtained with leurosine by therapy with levels ranging from 3.0–7.5 mg./Kg. (Table IV). A number of other transplantable tumors were also affected, including Freund, Ehrlich, and S-180 ascites tumors, B82A leukemia, and the Walker carcinosarcoma 256.

Extensive experimental evaluation indicated the "indefinite" survivors, obtained by administering certain of the crude extracts to animals implanted with the P-1534 leukemia, were not due to VLB or leurosine.

Continuing chemical investigation of those fractions (Table V), which gave high prolongation or indefinite survival of P-1534 animals, with such activity as a bioassay, led to the production of leurocristine and leurosidine (16). The activity of these alkaloids will be described in detail elsewhere, but prolongations ranging from

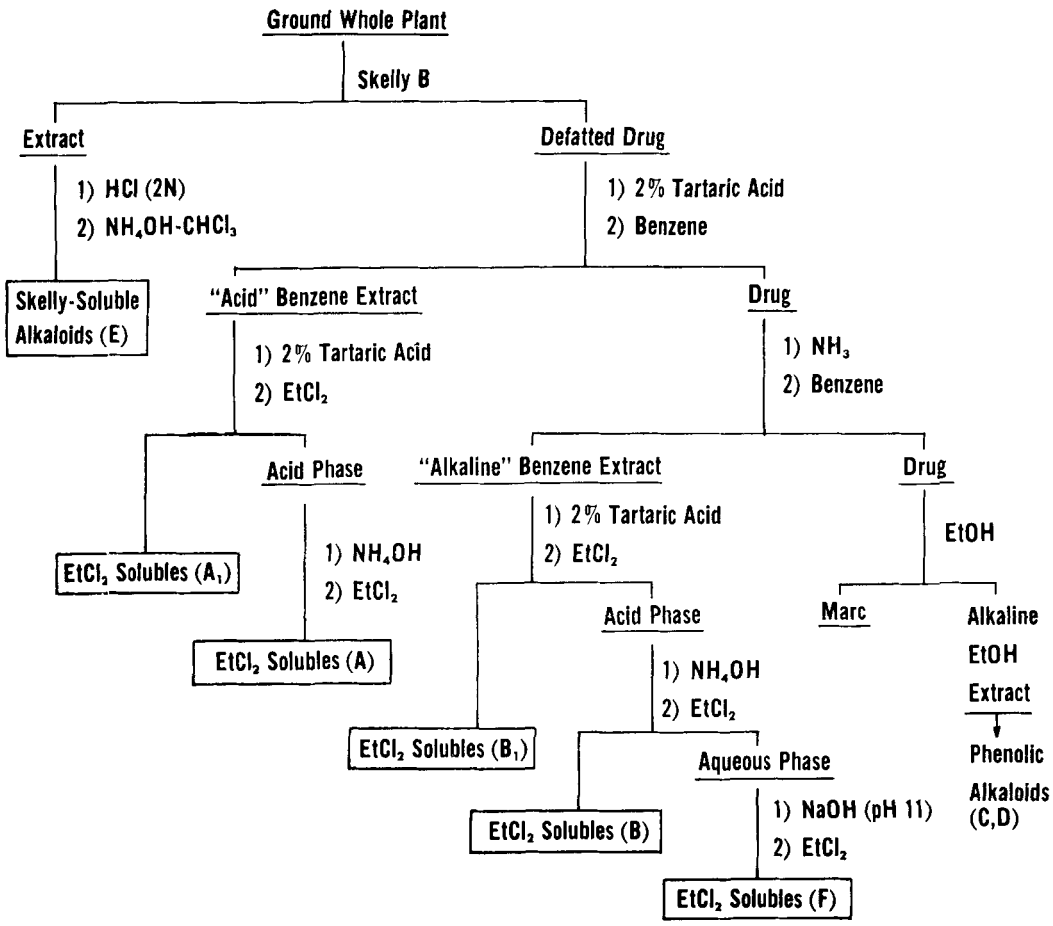


Fig. 1.—Extraction scheme.

30–220% over controls were obtained with leurocristine at levels from 0.05–0.35 mg./Kg., and 20–130% at levels from 2.0–10 mg./Kg. with leurosidine (Table VI) (35). At the higher levels of these ranges, 60–100% of the animals may be "cured" and survive indefinitely. The experimental tumor spectra of leurocristine and leurosidine are slightly broader than VLB and leurosine (Table VII).

With the exception of leurosine, all of these alkaloids may significantly prolong life of P-1534 animals, even when treatment is delayed until the animals are in near terminal states. In addition, in the case of leurocristine and leurosidine, very preliminary studies suggest that if such animals survive, they may become resistant to additional challenges with the same tumor. "Curing" of strain specific tumors in highly inbred strains of laboratory animals is in itself rare, but resistance to subsequent challenges of the same tumor is almost unique. It is unknown whether such reactions can be extrapolated usefully to any clinical situation, either concerning neoplasia or transplantation phenomena.

Pharmacological and toxicological studies with these alkaloids have not been published in detail. Vincaleukoblastine and leurocristine have been more extensively studied (36). Acute i.v. LD_{50}'s for VLB and leurocristine in mice are approximately 17.0 and 2.0 mg./Kg., respectively. Multiple doses of VLB caused marked leucopenia in rats and dogs at 0.50 and 0.1–0.2 mg./Kg., respectively, causing death in some instances. Leurocristine, leurosidine, and leurosine do not exert leucopenic effects in rats and dogs to this same degree. Transient rises in blood pressure of dogs receiving VLB or leurocristine have been observed. Leurocristine has also been observed to cause a slight paralysis of hind limbs in mice with a single i.v. administration of 2.0 mg./Kg. Other studies of possible neurological effects of these alkaloids are in progress.

Clinically, VLB has been more extensively studied than any of the other alkaloids. The

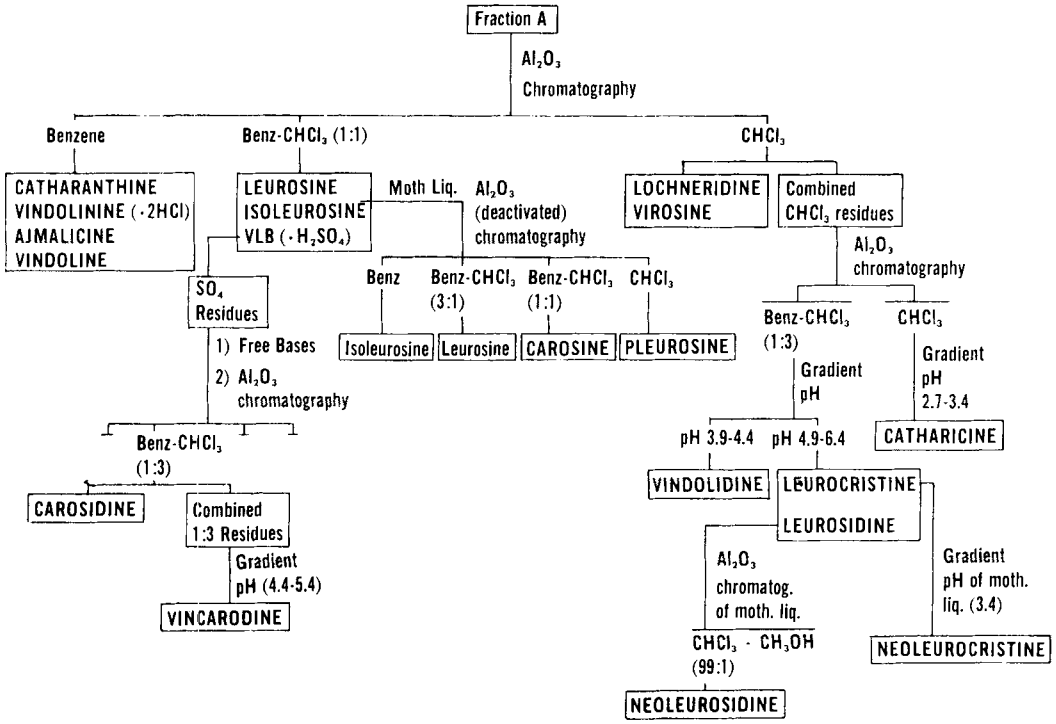


Fig. 2.—Flow scheme for alkaloids from fraction A.

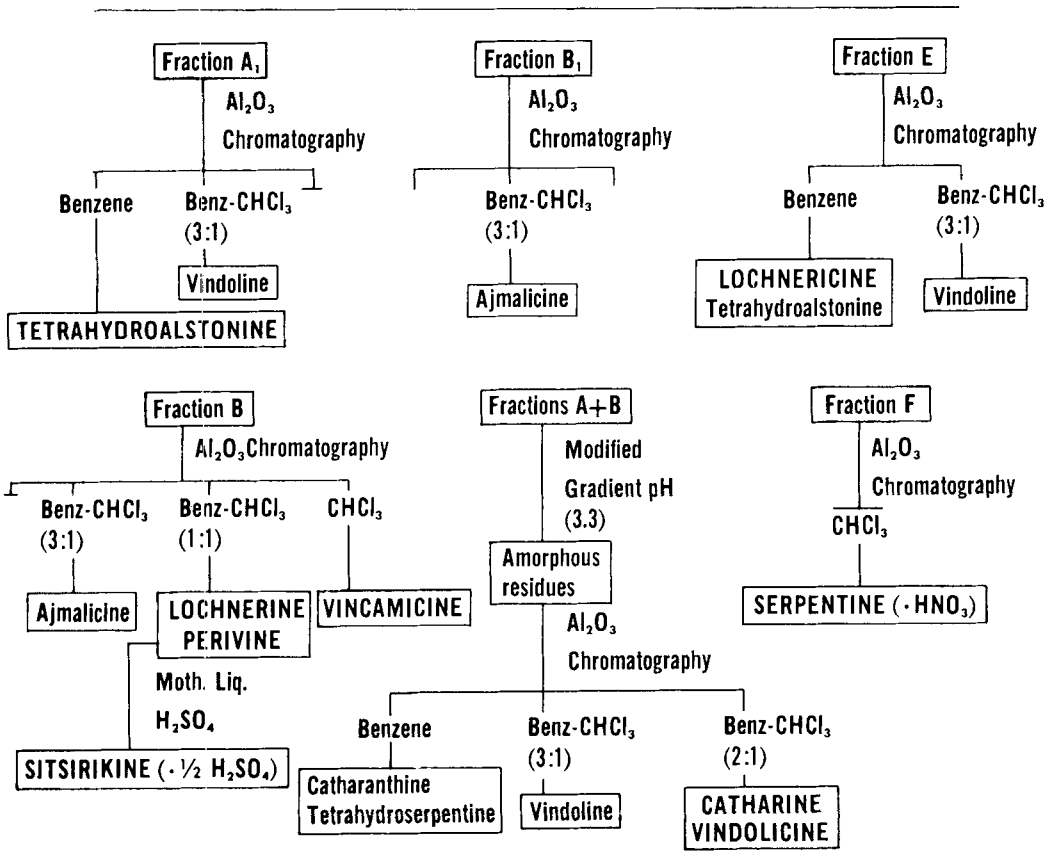


Fig. 3.—Flow schemes for alkaloids from fractions A_1, B_1, B, (A + B), E, and F.

TABLE III.—ACTIVITY OF ORIGINAL EXTRACT OF WHOLE PLANT AND CRUDE FRACTIONS AGAINST P-1534 LEUKEMIA

Material	Dosage, mg./Kg./day	Av. Wt. Change, Gm., T/C	Av. Survival Time, days, T/C	% Increase in Survival Time	% Indefinite Survival
Defatted whole plant	120.0	+0.1/+2.7	25.6/14.8	73	0
Fraction A	0.5	−0.6/+1.2	26.5/19.2	38	20
Fraction B	30.0	−1.2/+1.2	24.0/19.0	25	40
Total alkaloids	6.0	+0.8/+2.9	27.7/17.2	61	20
	7.5	+0.2/+0.6	29.8/13.4	122	0
	15.0	−2.3/+0.6	20.3/13.4	51	60
	15.0	+0.3/+0.5	30.0/13.0	130	0
	75 (Oral)	−1.5/+0.6	20.6/13.4	53	0

TABLE IV.—RESULTS OBTAINED WITH LEUROSINE AND VINCALEUKOBLASTINE TREATMENT OF THE P-1534 LEUKEMIA

Material	Dosage, mg./Kg./day	Av. Wt. Change, Gm., T/C	Av. Survival Time, days, T/C	% Increase in Survival Time
Leurosine	7.5	+0.2/+1.9	22.0/14.6	50
	3.0	+1.4/0.0	27.3/13.0	110
	6.0	+1.4/+1.3	26.4/17.2	53
	150.0 (Oral)	−1.2/+0.6	19.6/13.4	46
Vincaleukoblastine	0.05	+2.7/+0.7	23.0/16.2	41
	0.1	+1.7/+0.7	24.8/16.2	53
	0.3	+0.6/+0.9	22.8/13.4	70
	0.45	+0.3/+0.3	27.0/13.6	98
	0.45	+0.8/+0.6	30.6/13.2	131
	0.6	−1.7/−1.6	28.6/11.4	150
	0.6	−2.0/−1.6	26.0/11.4	128
	0.6	+0.3/−1.6	24.6/11.4	115
	1.5 (Oral)	−1.2/+0.7	27.6/16.2	70

most striking results of VLB therapy have been obtained in the treatment of Hodgkin's disease (37–41). Patients have been maintained in clinical remission for periods of over a year. Choriocarcinoma and carcinoma of the breast have also been treated effectively with this compound (42, 43). In all of these studies, beneficial results have been obtained even in tumors resistant to other commonly used chemotherapeutic agents.

A number of other types of tumors have also been observed to respond to VLB therapy. These include carcinomas of the bronchus and colon. Isolated instances of responses in seminoma, teratoblastoma, and melanoma have been reported.

Some investigators have obtained temporary remissions in various types of leukemia, including acute lymphocytic, acute monocytic, and myeloblastic. The remissions have not generally included complete bone marrow remissions. The results obtained with VLB in leukemias and tumors of the gastrointestinal and genitourinary tract do suggest the need for further evaluation in numbers of patients treated and types of treatment regimens.

Only the most preliminary clinical studies have been made with leurocristine. These reports suggest a striking percentage of complete hematological remissions in acute lymphocytic leukemia

TABLE V.—ANTI-P-1534 ACTIVITY OF AMORPHOUS FRACTIONS FREE OF VINCALEUKOBLASTINE AND LEUROSINE

mg./Kg.[a] XIX10	Toxic Deaths	Prolongation, %	Indefinite Survivors
3.0	0/5	?	5/5
3.0	0/5	?	5/5
3.0	2/5	?	3/5
0.6	2/5	181	0/5
18.0	2/5	147	0/5
4.5	0/5	151	1/5
9.0	1/5	147	2/5
9.0	0/5	174	1/5
6.0	0/5	123	4/5
20.0	0/5	148	1/5
30.0	0/5	238	4/5

[a] Intraperitoneally.

and responses in a spectrum of solid tumors (44–51). Proper placement of leurocristine in the clinical armamentarium against the oncological process must await further clinical study. At the present time there is no clinical information available concerning leurosine or leurosidine.

The mechanisms by which these agents exert antitumor effects are unknown. Tissue culture studies with VLB suggested an interference in metabolic pathways leading from glutamic acid to the citric acid cycle and to urea. Extension of these studies to experimental *in vivo* systems tend to confirm the *in vitro* results (15, 34, 52). Similar findings have also been made clinically (41, 53). Various abnormal mitotic figures including

TABLE VI.—ANTI-P-1534 ACTIVITY OF LEUROCRISTINE AND LEUROSIDINE

Material	mg./Kg.[a] XIX10	Toxic Deaths	Prolongation, %	Indefinite Survivors
Leurocristine	0.35	2/5	30	1/5
	0.30	2/5	?	3/5
	0.25	0/5	226	3/5
	0.20	1/5	110	2/5
	0.15	0/5	55	2/5
	0.12	0/5	49	0/5
	0.09	0/5	32	0/5
	0.06	0/5	24	0/5
Leurosidine	10.0	1/5	?	4/5
	7.5	0/5	?	5/5
	5.0	0/5	127	0/5
	4.0	0/5	75	0/5
	3.0	0/5	30	0/5
	2.0	0/5	21	0/5

[a] Intraperitoneally.

TABLE VII.—COMPARISON OF ANTITUMOR ACTIVITY OF FOUR "ACTIVE" ALKALOIDS

Tumor	Host	Alkaloids[a] A	B	C	D
Lilly mammary	DBA/1	+	...	...	+
Sarcoma 180	CAF[1]	...	...	...	...
Adenocarcinoma 755	C57B1/6	...	...	...	+
C-1498 Leukemia	C57B1/6	...	...	...	...
P-1534 Leukemia	DBA/2	+++	++	+++	+++
L-1210 Leukemia	DBA/2	...	...	...	...
Ridgeway osteogenic sarcoma	AKR	...	...	+++	+++
Mecca-lymphosarcoma	AKR	...	...	+	+
AKR Leukemia	AKR	...	+	...	++
Ehrlich ascites	Cox std.	+++	+++	...	...
Freund ascites	CAF[1]	+++	+++	++	++
S-180 Ascites	CAF[1]	+++	...	+	+++
B-82B Leukemia	C58	++	+	++	+++
Walker carcinosarcoma 256	Rat	+	+	+	...
Lilly mammary	C3H	...	...	...	...
Gardner lymphosarcoma	C3H	...	...	+	...
S-91 Melanoma	DBA/1	...	...	...	...
X-5563 Myeloma	C3H	N.D.	N.D.	++	+
High malignancy clone	C3H	N.D.	N.D.	...	...
Lilly rhabdomyosarcoma	Rat	+	...	...	..

[a] A, Vincaleukoblastine; B, leurosine; C, leurosidine; D, leurocristine. +, 30–50% Inhibition of solid tumors or prolongation of survival time in leukemias; ++, 50–100% inhibition of solid tumors or prolongation of leukemias; +++, 100% inhibition of solid tumors or >100% prolongation of leukemias; N.D., not done.

C-mitosis, ball metaphase, etc., have been obtained both *in vitro* and *in vivo* (15, 34, 52, 54). It seems unlikely that the therapeutic responses obtained are due to these cytological effects, since these are observed clinically and experimentally in tumors in the absence of any oncolytic effects.

Experimental studies in combination with other known cancer drugs, and the lack of cross resistance to them clinically, suggest mechanisms different than those of any such known compounds. In spite of the probable close structural relationships among these four alkaloids, there appears to be a lack of cross resistance and probable different mechanisms of action among them. These agents, regardless of mechanism, represent new and hitherto unknown leads towards effective chemotherapeutic treatment of a variety of human neoplasms.

CHEMISTRY

Prior to the discovery of the oncolytic properties of certain alkaloidal extracts prepared from the plant *Vinca rosea* Linn., a number of miscellaneous plant extracts had been reported to have some degree of antitumor activity (55). In several instances the pure chemical substance (s) had been isolated, *viz.*, podophyllin (56) and colchicine (57), and had been subjected to clinical testing.

Preliminary studies showed that the *Vinca rosea* Linn. alkaloids were not related to other naturally occurring oncolytic agents (12, 15) and that, by and large, they were indole and dihydroindole in character (58). Table I lists a number of alkaloids from this plant which had been described by earlier workers (14). They are all found in other genera and species of the *Apocynaceae*, and their structures are well

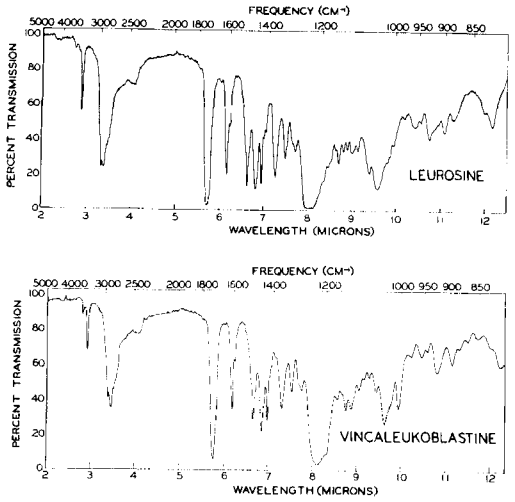


Fig. 4.—Infrared spectra of leurosine and vincaleukoblastine.

documented. The remaining alkaloids (Table II) were of unknown structure and it seemed pertinent, on the basis of the unique biological properties of certain of these alkaloids, to undertake a detailed study of their chemistry, with particular emphasis on leurocristine (16), VLB (59), leurosine (12), and leurosidine (16).

Investigation of the physical properties of leurosine and VLB (58) indicated that they were nonsymmetrical dimeric alkaloids. Titration showed the presence of two basic nitrogens, pK'a 5.5 and 7.0, one of which would quaternize when treated with methyl iodide or similar reagents. Analyses of the free bases, sulfates, dihydrochlorides, and quaternary salts indicated that they were $C_{46}H_{56-58}N_4O_9$ compounds (59). A study of the infrared spectra of the bases further supported this contention (thus the intensity of the indole N—H was approximately one-half that expected for a typical indolic C_{21} alkaloid) and indicated that VLB contained a hydroxyl group ($\lambda_{max.}^{CHCl_3}$ 2.8, 10.0 μ) that was not present in leurosine. Aside from this difference, the infrared spectra were very similar (Fig. 4) (58).

Comparison of these spectra with those of several other *Vinca rosea* alkaloids, notably catharanthine and vindoline (Table II), clearly indicated a close interrelationship between these compounds. When the infrared spectrum of a solution containing equimolar quantities of catharanthine and vindoline was compared to those of leurosine and VLB, it was virtually superimposable from 2–8 μ and quite similar up to 16 μ (Fig. 5). It was therefore assumed that the double molecules leurosine and VLB were composed of catharanthine and vindoline moieties, with minor molecular modification, bonded together in some unique manner (58). With this thought in mind, we turned our attention to the study of these two smaller molecules.

Catharanthine (I) was found to be a $C_{21}H_{24}N_2O_2$ compound and to form a methiodide readily. The physical characteristics of the quaternary salt showed that the basic nitrogen (pK'a 7.0) was analogous to the one forming the salt in the dimeric compounds. The ultraviolet spectrum was typical of an unsubstituted indole, while the infrared spectrum showed indole N—H (2.9 μ), carbomethoxyl moiety (5.78, 8.05 μ), and a triplet at 6.8 μ, characteristic of the Iboga-type alkaloids. The presence of a single double bond was seen from hydrogenation studies which yielded dihydrocatharanthine (II), thereby indicating that the parent base was pentacyclic (24).

Treatment of dihydrocatharanthine (II) under reflux with hydrazine in absolute ethanol, or saponification followed by brief acid treatment, resulted in decarboxylation in a manner analogous to that reported for voacangine (III) and related alkaloids (60, 61).

The new descarbomethoxy base, epi-ibogamine (IV), $C_{19}H_{24}N_2$, was further characterized as the hydrochloride $C_{19}H_{24}N_2 \cdot HCl$ (62). The analogous reaction on catharanthine (I) was unsuccessful; however, by the use of vigorous conditions (refluxing in concentrated hydrochloric acid) a poor yield (*ca.* 1%) of descarbomethoxycatharanthine (V) could be obtained. This compound was hydrogenated (platinum dioxide in alcohol) and afforded epi-ibogamine (IV). The failure of catharanthine to decarboxylate readily may be explained if the position of the double bond is as shown in (I). The corresponding intermediate for this type of reaction (60, 62) would require the highly strained structure shown in (VI).

The position of the carbomethoxyl at C-18 was also shown by the formation of the tetrahydro-1, 3-oxazine (VII) of catharanthinol (VIII), (the lithium aluminum hydride reduction product of I). This derivative was obtained by the treatment of catharanthinol with dry hydrogen chloride in acetone (63). As expected, the compound does not have any indole N—H or OH absorption in the infrared spectrum; $C_{23}H_{28}N_2O_2$ (62).

The relationship of catharanthine (I) and its derivatives to the corresponding Iboga alkaloids [*e.g.*, ibogamine (IX) and coronaridine (carbomethoxyibogamine) (X)] was indicated from the similarities of their infrared spectra and

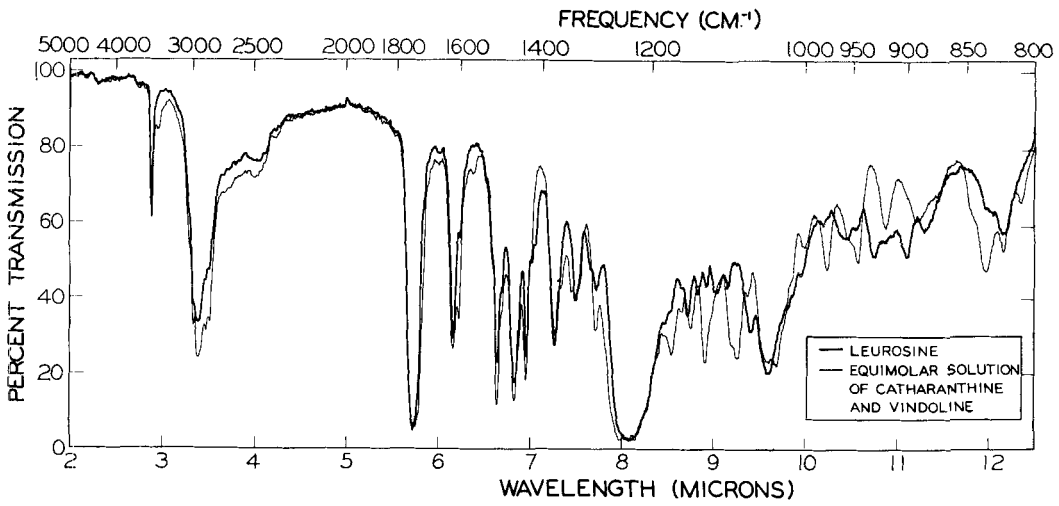
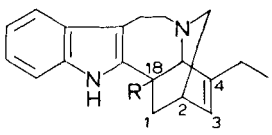
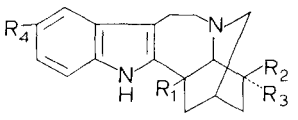


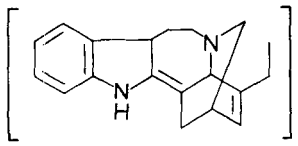
Fig. 5.—Infrared spectra of leurosine *vs.* an equimolar solution of catharanthine and vindoline.



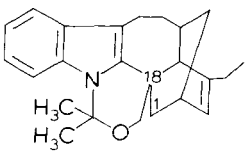


I R = $COOCH_3$
VIII R = CH_2OH
V R = H

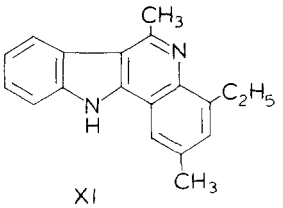
II R_1=$COOCH_3$; R_2=H; R_3=C_2H_5; R_4=H; Dihydrocatharanthine
III R_1=$COOCH_3$; R_2=C_2H_5; R_3=H; R_4=OCH_3; Voacangine
IV R_1=H; R_2=H; R_3=C_2H_5; R_4=H; Epi-ibogamine
IX R_1=H; R_2=C_2H_5; R_3=H; R_4=H; Ibogamine
X R_1=$COOCH_3$; R_2=C_2H_5; R_3=H; R_4=H; Coronaridine
XIII R_1=H; R_2=C_2H_5; R_3=H; R_4=OCH_3; Ibogaine



VI



VII



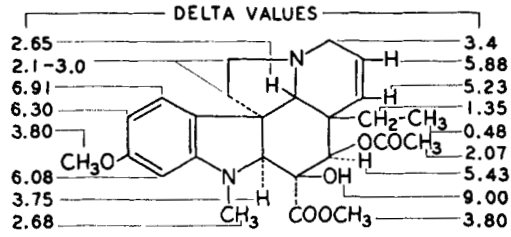
XI

finally proved by the isolation of the indoloquinoline (XI) (64) from the selenium dehydrogenation of epi-ibogamine (IV). This product (XI) was found to be identical in every respect (X-ray powder pattern, infrared, ultraviolet, and mass spectra) with an authentic sample obtained by dehydrogenation of ibogamine (IX) (64).

The position of the endocyclic double bond was indicated by the NMR spectrum of catharanthine (I) which has shown the presence of an ethyl group (methyl triplet centered at 1.10 δ with peaks separated by 7 c.p.s.) and a single proton on a double bond (peak at 5.90 δ). The position of the double bond was corroborated by products resulting from dehydrogenation of catharanthine (I) and dihydrocatharanthine (II), using palladium on carbon (62).

The former gave a good yield of 3-ethyl pyridine (XII) at 150–160°, while the latter required a temperature of 230–250° to afford 3-methyl-5-ethyl pyridine in a manner similar to that previously described for voacangine (III) (65). The formation of (XII) is explained by the fission of the C-1–C-2 allylic bond, while in dihydrocatharanthine (II) the bond breaks as in voacangine (III) between C-1 and C-18.

The hydrogenation of catharanthine led to



XIV

only one isomer (*vide supra*). From the models of (I), it is apparent that the hydrogen must come in from the side nearest to N_b to give the axial ethyl group shown in (II). Therefore, dihydrocatharanthine (II) and epi-ibogamine (IV) are epimeric at C-4 with coronaridine (X) and ibogamine (IX), respectively, since the C-ethyl group in the Iboga alkaloids, *e.g.*, ibogaine (XIII) has been shown to be equatorial (66), and catharanthine must be Δ^3-dehydrocoronaridine (62).

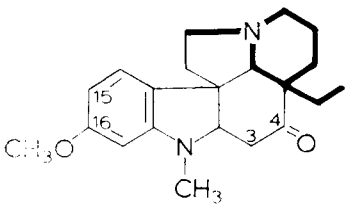
With this information in hand, attention was turned to the structure elucidation of vindoline. Vindoline (XIV), the most abundant alkaloid in the leaves, was found to be a $C_{25}H_{32}N_2O_6$ dihydroindole compound (24). Its basic nitrogen did not form a methiodide in analogy to the nitrogen pK'a 5.4 in the dimeric alkaloids. The nature of the six oxygens was determined by the formation of suitable derivatives. Thus, treatment of vindoline with acid (8 min. reflux—N_2) afforded desacetylvindoline (XV), $C_{23}H_{30}N_2O_5$, corresponding to the loss of an acetyl group. This was further corroborated in the infrared spectrum by the appearance of a hydroxyl band (2.7 μ) and corresponding reduction of intensity of the carbonyl band (24).

The presence of the methyl ester was shown by lithium aluminum hydride reduction of the base with simultaneous removal of the acetyl to yield vindolinol, $C_{22}H_{30}N_2O_4$ (24). The infrared spectrum of vindoline, in which a broad band at 3.5–4.0 μ was conspicuous, showed that the fifth oxygen was a hydrogen-bonded hydroxyl. Formation of the diacetate $C_{27}H_{34}N_2O_7$ (acetic anhydride in pyridine) was accompanied by the disappearance of this broad band in the infrared spectrum (24). From the study of the ultraviolet and infrared spectra, the sixth oxygen was shown to be present as an aromatic methoxyl on a dihydroindole chromophore. The confirmation of the assignment of the oxygen functional groups was obtained from the NMR spectra (XIV) (Fig. 7) (67). Vindoline consumed one mole of hydrogen at atmospheric pressure, showing it to be a pentacyclic compound and afforded dihydrovindoline (XVI) which could be converted to an amorphous hygroscopic hydrochloride. Pyrolysis of this salt at 195–200° in vacuum gave a distillate from which a $C_{21}H_{28}N_2O_2$ compound (XVII) was obtained in 15% overall yield by direct crystallization from hexane. The methoxydihydroindole portion of this compound was the same as in vindoline (XIV), and the second oxygen was found to be present as a ketonic carbonyl ($\lambda_{max.}^{CHCl_3}$ 5.85 μ) (67).

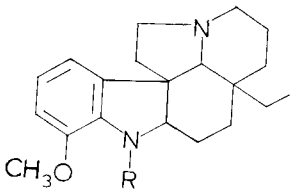
Careful comparison of the mass spectra of the pyrolysis ketone (XVII) and dihydrovindoline (XVI) with that of N-methyl desacetyl aspidospermine (XVIII) (68) indicated the presence of the latter ring system in vindoline and its derivatives, since in all three compounds identical intense peaks were found at m/e 124, 174, 188, and 298 (68).

The position of the carbonyl in (XVII) was assigned on the basis of the presence of the peak at m/e 298 (M-42) (XXII) which suggested that this group involved either C-3 or C-4. Position 4 was selected for two reasons: the typical ABX pattern at low field (3 to 3.5 δ) in the NMR spectrum (also consistent with structure XVII) indicated three protons between the carbonyl group and nitrogen; and equilibration with CH_3OD/methoxide resulted in the introduction of only two deuterium atoms per molecule (M = 342). The peak at m/e 124 (XIX) comprises the atoms outlined by heavy lines in structure (XVII) while the 174 (XX) and 188 (XXI) peaks contain the dihydroindole moiety (as indoles) with one or two carbons, respectively, of the "tryptophan" dimethylene bridge attached. The fragment of mass 298 (XXII) involves the loss of the two-carbon bridge (C-3 and C-4) containing the oxygen functions (except the aromatic methoxyl) of each derivative. The only other major peak in the ketone occurs at m/e 166 (XXIII) which shifts to m/e 168 in the deutero derivative. Structure (XXIII) is assigned to it since this peak corresponds to fragment (XXIV) of mass 152 (69) in the spectrum of aspidospermine (XXV). The fragmentation is more complex in dihydrovindoline, giving rise to a series of peaks at m/e 398, 311, 284, and 224 in addition to those already mentioned. The proposed structure for vindoline is consistent with the formation of these additional ions.

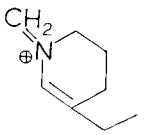
The position of the aromatic methoxyl was shown to be at either C-15 or C-16 by examination of the typical 1, 2, 4 aromatic proton pattern (*ortho* splitting J = 8 c.p.s., *meta* splitting J = 2.5 c.p.s.). The final selection of C-16 is based on the comparison of the infrared and ultraviolet spectra of vindoline with 6-methoxy-N-



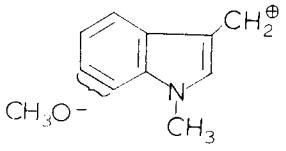
XVII



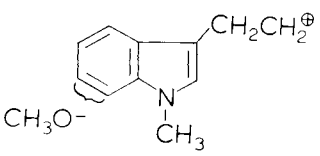
XVIII R = CH_3 O
XXV R = CH_3C–



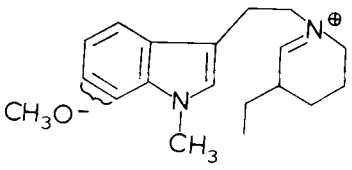
XIX



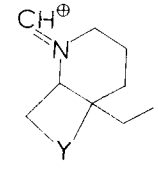
XX



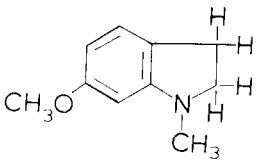
XXI



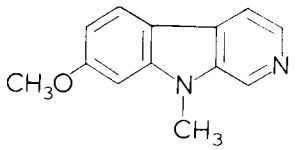
XXII



XXIII Y = C=O
XXIV Y = CH_2



XXVI

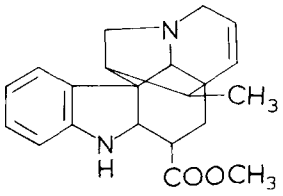


XXVII

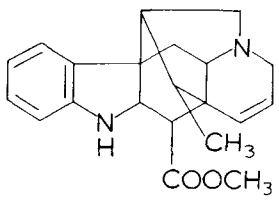
methyldihydroindole (XXVI) and by the isolation of *ind*-N-methylnorharmine (XXVII); m.p. 112–114°, hydrochloride m.p. 232–236°, molecular weight (M) 212 (by mass spectrometry), $\lambda_{max.}^{EtOH}$ 243 (37,500), 303 (15,100), 336 (4,300), from a soda lime distillation of vindoline at 325°. This derivative also indicates the position of the N-CH_3 as being on the anilino nitrogen. A second product (XVII) which was identical to the C_{21} ketone obtained above was also isolated from the soda lime reaction. Examination of the NMR spectrum of vindoline (XIV) (Fig. 7) allows the assignment of each proton as shown. The following assignments, however, deserve some comment. The unsplit peak of the proton on the carbon bearing the acetoxyl moiety shifts to 4.07 δ in desacetyl vindoline (XVIII). The protons of the methylene portion of the C-ethyl group are not equivalent and are found as a 12-band multiplet centered at 1.35 δ (J = 7.5 c.p.s.). The methyl triplet, however, is almost symmetrical in vindoline, being found at an exceptionally high field 0.48 δ (J = 7.5 c.p.s.) for a grouping of this type. This high field appears to be due to increased shielding by ring currents from the aromatic ring. The coupling constant of the two *cis* vinyl protons is J = 10 c.p.s., and one of these (5.88 δ) is further split by two non-equivalent adjacent protons (3.4 δ) with coupling constants J = 5 and 2 c.p.s. (67).

The mass spectral evidence, besides indicating the ring system of vindoline, shows that the oxygen functions other than the aromatic methoxyl can only be present at positions 3 and 4, and therefore must be arranged as shown in structure (XIV) to be consistent with NMR data. The formation of the ketone is also easily explained from this formulation (dehydration, hydrolysis, decarboxylation) (67). The stereochemistry of vindoline is as yet tentative and rests on evidence which will be presented elsewhere.

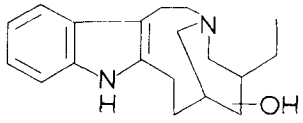
It is interesting to note that vindolinine (24, 70), another *Vinca* alkaloid with a slightly modified aspidospermine (XXV) skeleton, contains a double bond at the same position as vindoline (XIV). The structure of this compound is most probably best represented by (XXVIII) or (XXIX) and was arrived at by a joint effort of Djerassi's group at Stanford, Le Men and coworkers in Paris, and the writers at the Lilly Laboratories (71). The structural assignment of this compound is unique in that it is based largely on interpretation of mass spectrometric fragmentation patterns, combined with NMR measurements, without utilization of classical chemical degradations. In all, over 30 derivatives were studied, and these were obtained by the suitable use of acetylation, reduction, and acylation reactions (71).



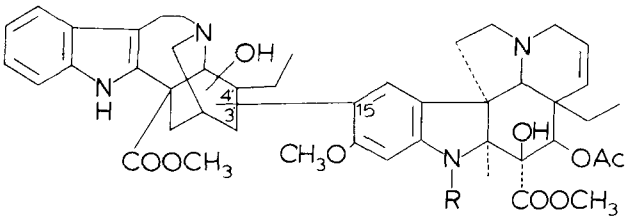
XXVIII



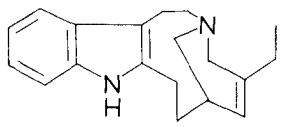
XXIX



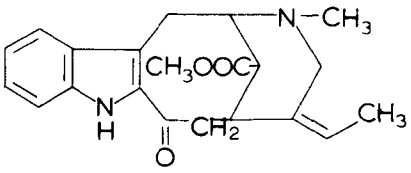
XXXIV



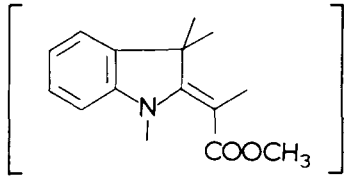
XXX R = CH_3
XXXIII R = CHO



XXXV



XXXVII



XXXVIII

As mentioned above, spectral evidence strongly suggested that the dimeric alkaloids were composed of a molecular combination of catharanthine (I) and vindoline (XIV). This assumption could be further substantiated by the products of acid cleavage (concentrated hydrochloric acid, stannous chloride, tin, reflux) carried out on the dimeric alkaloids. In each case there was obtained upon chromatography of the reaction mixture an indole compound (*vide infra*), followed by vindoline derivatives. Vincaleukoblastine (XXX) and leurosine (XXXI) afforded desacetylvindoline (XV), thus proving the identity of the dihydroindole portion of these alkaloids. The corresponding fraction from the cleavage of leurocristine yielded des-N(*a*)-methyldesacetylvindoline (XXXII), M = 400, $C_{22}H_{28}N_2O_5$. The mass spectrum, as well as ultraviolet and infrared spectra, demonstrate the relationship of this compound to desacetylvindoline. Both VLB (XXX) and leurocristine (XXXIII) yield the same tetracyclic indole derivative velbanamine (XXXIV), $C_{19}H_{26}N_2O$, the infrared spectrum of which clearly shows the oxygen to be present as a hydroxyl (72).

A related tetracyclic indole derivative, cleavamine (XXXV), $C_{19}H_{24}N_2$ was obtained from the cleavage of leurosine. (The correct formula of leurosine is still in doubt due to difficulty of preparing a solvent-free sample.) Isolation of cleavamine demonstrates the relationship of the indole portion of C_{46} alkaloids to catharanthine (I) since it was also obtained by a similar acid treatment of the latter. Cleavamine is believed to possess structure (XXXV) and velbanamine appears to be its dihydrohydroxy derivative (XXXIV). The mass spectra indicate the same carbon skeleton for the two compounds and are consistent with the proposed structures (72).

The cleavage reaction demonstrates that leurocristine (XXXIII) differs from VLB (XXX) only in the vindoline portion of the molecule. The nature of this difference was demonstrated in the following manner.

Analyses of leurocristine base (XXXIII), sulfate, and monomethiodide, are consistent with a formulation of $C_{46}H_{54}N_4O_{10}$. Its ultraviolet spectrum $\lambda_{max.}^{EtOH}$ 220 mμ (log a_M 4.65), 255 mμ (log a_M 4.21), 296 mμ (log a_M 4.18) and $\lambda_{max.}^{EtOH}$ 275 mμ (log a_M 4.02) is quite different from that of VLB (XXX) and also indicates a different substitution on $N_{(a)}$ of the dihydroindole moiety (16).

The infrared spectra of VLB and leurocristine (Fig. 6) are quite similar with the exception of the presence of a strong additional band, $\lambda_{max.}^{CHCl_3}$ 5.94 μ in the spectrum of the latter. The NMR spectra differ in that the N—CH_3 proton resonance at 2.73 δ present in VLB is missing in leurocristine; and, conversely, in place of only one low field, 9.8 δ proton in VLB, there are two in leurocristine at 9.5 and 8.9 δ.

Lithium aluminum hydride reduction of the two alkaloids afforded good yields of the same pentahydroxy derivative, $C_{42}H_{54}N_4O_6$ (XXXVI). This formulation is consistent with the reduction of two methyl esters, one acetate and an *N*-formyl group in the case of leurocristine. The NMR spectrum of (XXXVI) shows, accordingly, only two methyl signals, aromatic OCH_3 at 3.8 δ and N—CH_3 at 2.73 δ. The N—CHO of leurocristine is therefore at the anilino nitrogen in place of the N—CH_3 in VLB and further, since the indole cleavage products (velbanamine) are identical, then leurocristine is des-$N_{(a)}$-methyl $N_{(a)}$-formyl VLB (72).

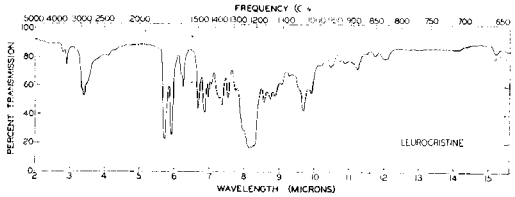


Fig. 6.—Infrared spectrum of leurocristine.

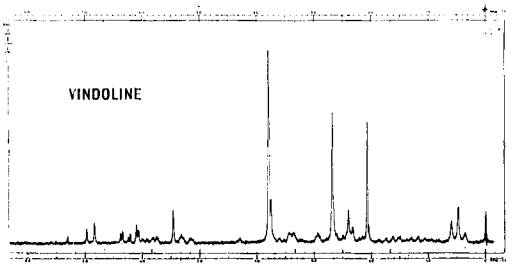


Fig. 7.—NMR spectrum of vindoline. Low field-OH not shown; peak at 7.32δ due to chloroform.

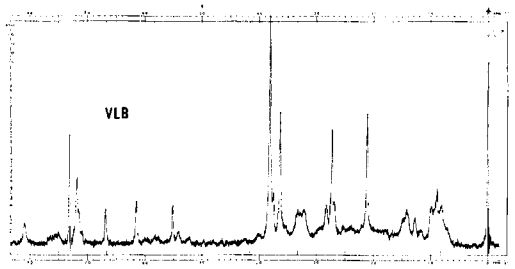


Fig. 8.—NMR spectrum of VLB. Low field-OH not shown; peak at 7.32δ due to chloroform.

The mode of attachment of the indole portion of the dimeric alkaloids to the dihydroindole fragment must be through the C-15 aromatic carbon since the NMR spectrum shows only two protons in a 1 : 4 relationship in the aromatic portion of the molecule rather than the 1, 2, 4 three-proton pattern present in vindoline (Figs. 7 and 8).

These data clearly indicate the partial structures (XXX) and (XXXIII) for VLB and leurocristine, respectively. On the basis of biogenetic considerations (free radical photosynthesis), as well as the mode of cleavage of these alkaloids, we are prompted to propose either C-3′ or C-4′ as the most likely site of attachment in the indole moiety and possible positions of the tertiary hydroxyl. The elucidation of this problem is at present in progress in these laboratories.

Little is known of the chemistry of the remaining alkaloids in Table II; however, they can be classified into three general groups. A number of the bases are closely related to the VLB-leurosine group of alkaloids (1, 27). The resemblance is seen in their infrared, ultraviolet, and NMR spectra, as well as from some results of acidic cleavage of several of these. Thus, leurosidine and isoleurosine both yield desacetylvindoline and an as yet unidentified indole derivative when treated as described above. The members of this group are listed in Table VIII.

The second group consists of monomeric alkaloids which range from C_{19} to C_{25} compounds (Table IX).

The infrared and ultraviolet spectra of perivine indicate that it is closely related to vobasine (XXXVII) (73), a 2-acyl indole alkaloid.

TABLE VIII.—VLB-LEUROSINE-LIKE ALKALOIDS

1. Vincaleukoblastine
2. Leurosine
3. Leurocristine
4. Leurosidine
5. Isoleurosine
6. Catharine
7. Carosine
8. Pleurosine
9. Catharicine
10. Neoleurocristine
11. Neoleurosidine

TABLE IX.—MONOMERIC ALKALOIDS

1. Perivine
2. Virosine
3. Catharanthine
4. Vindoline
5. Lochnericine
6. Vindolinine
7. Lochneridine
8. Sitsirikine

Perivine also contains an ethylidene group, N-methyl ester, and a 2-acyl indole chromophore.

Sitsirikine appears to be an as yet undescribed yohimbine isomer.

The remaining alkaloids are present in trace quantities and little is known about them. Lochnericine and lochneridine are related and possess the chromophore shown in (XXXVIII).

The third group consists of dimeric alkaloids which are not indole-indoline in nature (Table X).

TABLE X.—MISCELLANEOUS DIMERIC ALKALOIDS

1.	Vindolicine
2.	Vindolidine
3.	Vincamicine
4.	Vincarodine
5.	Carosidine

Of these only the first two are fairly well characterized, and they appear to be symmetrical dimers of vindoline.

Vincarodine appears to be related to vincine obtained from *V. minor* Linn. (74, 75).

Further work is planned in these laboratories toward structural elucidation of these alkaloids, with particular emphasis being placed on the interrelationships of the growing number of indole-indoline compounds.

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Stability of Cyanocobalamin in Liver Preparations for Use in the Treatment of Pernicious Anemia

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A deficiency of vitamin B_{12} has been found in a significant number of commercially available injectable products containing liver extract with added cyanocobalamin, with or without other added ingredients. In a study of the stability of cyanocobalamin when added to liver injectable preparations, with and without added folic acid, mixtures were stored in the dark at room temperature and examined at intervals throughout 1 year. No appreciable loss in cyanocobalamin occurred during the first month. Solutions stored at pH 5.0 were relatively stable for 12 months. At pH 6.5, destruction occurred within 3 months in some samples. Greater destruction occurred when the pH was raised to 8.0. Progressive destruction was observed in unstable samples after 6- and 12-month periods. With some preparations, destruction increased when folic acid was present, and in some instances when solutions were in contact with rubber stoppers.

CYANOCOBALAMIN in aqueous preparations has been found to be more stable than the hydroxo analog (1, 2) and, also, more stable in the absence of its analogs (3). Its stability in solutions decreased under certain conditions in the presence of other water-soluble vitamins (4–12) and in the presence of degradation products of these other vitamins (9, 13–15). The effect of impure phenol (16), other components of aqueous solutions (2, 17–21), light (22, 23), and oxygen (4, 24) on the stability of vitamin B_{12} also has been reported.

Procedures for predicting the stability of vitamins, including vitamin B_{12}, have been devised (25, 26) which involve studies at elevated temperatures. A report of studies on oral multivitamin preparations revealed that potencies of several vitamins, including vitamin B_{12}, were markedly affected by shelf age and storage conditions for certain types of products (27). The authors called attention to the responsibility of the manufacturer for maintenance of potency of these products throughout normal shelf life, and suggested that an expiration date on the label would be helpful in ensuring that a product meets its label claim.

Despite the availability of much information relating to the stability of vitamin B_{12} in various pharmaceutical preparations, that dealing with the stability of cyanocobalamin when added to liver solutions is limited.

There are on the market injectable preparations containing liver extract with added cyanocobalamin that are labeled for use in the treatment of pernicious anemia. Some of these products also contain added minerals, folic acid, and other vitamins. It has been shown conclusively in this laboratory that cyanocobalamin is unstable in many of the products with labeled potencies varying from about 10 to 120 mcg. of vitamin B_{12} per ml. It is not unusual to find deficiencies of vitamin B_{12} ranging from 20 to 50% and, in some instances, as great as 90% or more. A marked difference may occur in the degree of deterioration among vials bearing the same code. Deterioration frequently progresses during storage, even at 10°. We also have found that when a 1- or 2-ml. portion has been withdrawn from a 10-ml. multiple-dose vial, the vitamin B_{12} potency in the remainder may decrease as much as 25% after a 1-week storage period at 10°. Gakenheimer (16) has called attention to the influence of liver solutions upon the stability of added cyanocobalamin and pointed out that in a given liver extract which may retain its vitamin B_{12} activity for 1 year, added cyanocobalamin may not be stable. He emphasized that liver solutions must be pretested for compatibility when vitamin B_{12}, with or without folic acid, is to be added.

Our findings led us to carry out a study on the stability of cyanocobalamin when added to liver solutions, with and without the addition of folic acid.

Received November 15, 1961, from the Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biological and Physical Sciences, Division of Nutrition, Washington, D. C.

Accepted for publication November 27, 1961.

Presented in part to the Association of Official Agricultural Chemists, Washington, D. C., meeting, October 1961.

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EXPERIMENTAL

Assay Methods

Vitamin B_{12}.—The A.O.A.C. (microbiological) method (28) was used. There was good agreement between the microbiological results and those obtained by the U.S.P. (spectrophotometric) method (29), slightly modified, on the cyanocobalamin stock solution and the control samples of cyanocobalamin and cyanocobalamin-folic acid.

Folic Acid.—The U.S.P. (chemical) method (30), slightly modified, was used. There was good agreement between the chemical results and those obtained spectrophotometrically on the folic acid stock solution and the control samples of cyanocobalamin-folic acid.

Stock Solutions

Cyanocobalamin and folic acid were of U.S.P. XV grade and all reagents were of analytical grade. Distilled water used as a solvent had a specific conductance of 0.5 micromho. Sterile solutions were used and all operations (including the subsequent preparation of sample solutions and filling and stoppering of vials) were conducted under aseptic conditions.

Liver, cyanocobalamin, and folic acid solutions were buffered with 0.05 *M* sodium acetate. Redistilled phenol was used at 0.5% concentration as the bacteriostatic agent in the preparation of each solution since it was already present in the liver solutions.

Acetate Buffer.—A 0.05 *M* solution of sodium acetate was adjusted to pH 6.5 with acetic acid.

Liver.—Solutions were taken from intact vials of U.S.P. XV grade liver injection and liver injection crude. These solutions were from various lots of samples that had accumulated in the laboratory and represented preparations from a number of manufacturers. Selection of the lots was arbitrary and stock solutions prepared from these lots did not represent any one manufacturer's product, and also were not necessarily identical with solutions used commercially. Each of six stock solutions was prepared by compositing three different lots of liver preparations and adjusting the slightly acidic solutions to pH 6.5 with sodium hydroxide solution.

Cyanocobalamin.—This solution, adjusted to pH 6.5 with acetic acid, contained 150 mcg. of cyanocobalamin per ml.

Folic Acid.—This solution contained the equivalent of 15 mg. of folic acid per ml. Folic acid was suspended in water, sodium hydroxide solution added until solution was complete (about pH 8.0), and adjustment to pH 6.5 was made with acetic acid.

Sample Solutions

At pH 6.5.—This pH was selected because it was near the value we had obtained for many liver-cyanocobalamin injectable solutions examined, some of which also contained folic acid. Most of the samples examined were within a pH range of 6.2–6.9, with an extreme range of 5.0 to 9.0.

(*a*) *Liver.*—Each of the six samples consisted of a portion of the respective liver stock solution.

(*b*) *Cyanocobalamin Control.*—Stock solutions of acetate buffer and cyanocobalamin were mixed in the ratio of 2:1.

(*c*) *Cyanocobalamin-Folic Acid Control.*—Stock solutions of acetate buffer, cyanocobalamin, and folic acid were mixed in the relation of 1:1:1.

(*d*) *Liver-Cyanocobalamin.*—Stock solutions of acetate buffer, liver, and cyanocobalamin were mixed in the relation of 1:1:1.

(*e*) *Liver-Cyanocobalamin-Folic Acid.*—Stock solutions of liver, cyanocobalamin, and folic acid were mixed in the relation of 1:1:1.

At pH 5.0.—A portion from the control under (*b*) and from each of samples 1, 3, and 6 under (*d*) was adjusted to pH 5.0 by the addition of acetic acid (about 0.3 ml./100 ml. solution).

At pH 8.0.—Portions similar to those taken for pH 5.0 adjustment were adjusted to pH 8.0 by the addition of 0.5 *M* tribasic sodium phosphate (about 0.2 ml./100 ml. solution).

Procedure

An 11-ml. portion of the respective sample or control solution was added to each vial used in the different phases of the study and the vial sealed immediately with a rubber stopper. Vials were Kimble[1] serum bottles, Neutraglas, narrow mouth, short form, No. 14196 for the 10-ml. size, and No. 14200 for the 20- and 50-ml. sizes. The respective volume of air in the 10-, 20-, and 50-ml. vials was 3, 16, and 50 ml. The 20- and 50-ml. vials were used to determine if any increase in the amount of enclosed air affected the stability of the cyanocobalamin control solution and the liver-cyanocobalamin sample solutions 1, 3, and 6 when stored at pH 6.5. Rubber stoppers were West[2] No. 124 (pink) stock. They were washed in a warm solution of sodium lauryl sulfate and rinsed repeatedly with distilled water. They were then immersed in distilled water, autoclaved, and rinsed. This latter process was repeated. A sufficient number of vials was prepared so that after portions were removed for assay, the remaining contents of an entered vial were discarded.

Determinations were made of the initial vitamin B_{12} and folic acid potencies of the vial contents from each series of solutions under study. Thereafter, determinations were made at intervals of 1, 3, 6, and 12 months with the vials stored upright so that the rubber stoppers were never in contact with the solutions. Storage was in the dark at 23.5 ± 1.5°. One set of vials for each phase of the study was stored in an inverted position to determine if potency was affected by contact of the solution with the vial stopper. These were examined only at the end of the 12-month storage period.

RESULTS

Liver Solutions.—Considerable destruction of vitamin B_{12} occurred in four of the six samples during twelve months storage in vials in upright position as shown in Table I. In fact, some loss was apparent at the end of the first month. Two of these unstable solutions were appreciably affected by contact with the stopper in the inverted vials.

Cyanocobalamin in Control Solutions.—There was no loss of cyanocobalamin activity in the control solutions throughout the 12-month storage period

[1] Kimble Glass Co., Subsidiary of Owens-Illinois, Toledo 1, Ohio.
[2] West Co., Phoenixville, Pa.

TABLE I.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
1	1.2	1.2	1.2	1.2	1.2	1.2
2	2.1	2.1	2.1	1.8	1.8	1.8
3	2.7	2.3	2.3	2.2	2.1	1.7
4	3.6	3.1	2.8	2.7	2.1	2.2
5	8.6	7.4	6.8	5.9	4.4	4.2
6	11.1	9.6	9.6	7.8	6.6	5.0

[a] Stored in 10-ml. vials in the dark at room temperature.

(Tables II–V). Cyanocobalamin alone in aqueous solution was stable at pH 5.0, 6.5, and 8.0; at pH 6.5 in the presence of folic acid; and in all solutions in contact with vial stoppers.

Liver Solutions with Cyanocobalamin.—Stability of vitamin B_{12} in the six samples stored at pH 6.5 in 10-ml. upright vials is shown in Table II. No appreciable destruction of vitamin B_{12} occurred during the first 3 months. At the end of 6 months, there was a loss in potency of 10%, or more, in four samples. Three of these samples continued to lose potency during the next 6 months. Vitamin B_{12} in the four unstable solutions was markedly affected by contact with the stopper.

TABLE II.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	50	50	49	49	50	50
1	50	50	47	45	38	23
2	51	51	49	45	44	32
3	51	51	48	42	37	19
4	51	51	49	45	40	26
5	53	52	53	50	49	45
6	54	53	52	50	50	47

[a] Stored in 10-ml. vials in the dark at room temperature.

It is shown for the three samples listed in Table III that the increase in amount of air enclosed in vials has no deleterious effect upon vitamin B_{12}. The results obtained on these samples correspond closely with those from the same three samples contained in 10-ml. vials included in Table II.

There was a marked change in the stability of two of these same three solutions stored in 10-ml. vials when the pH was altered, as shown in Table IV. At pH 5.0, the loss of vitamin B_{12} was 10% or less in samples 1 and 3, even when stored 12 months in contact with the vial stopper. The loss was drastic, however, in these two samples when the pH was raised to 8.0. The greatest stability occurred in sample 6. Even when inverted for 12 months, at pH 8.0, the loss in vitamin B_{12} was only 15%.

Liver Solutions with Cyanocobalamin and Folic Acid.—During the first month, as shown in Table V, there was little effect upon stability of vitamin B_{12} from the addition of folic acid to the six samples stored at pH 6.5 in upright 10-ml. vials. A loss in

TABLE III.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 6.5[a]

Sample No.	Vial Size, ml.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	20	50	50	50	50	50	49
	50	50	50	50	50	50	50
1	20	50	49	47	44	38	23
	50	50	49	47	45	38	23
3	20	51	51	48	43	38	18
	50	51	50	47	43	37	19
6	20	54	53	52	49	49	48
	50	54	53	52	50	49	47

[a] Stored in 20- and 50-ml. vials in the dark at room temperature.

TABLE IV.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 5.0 and 8.0[a]

Sample No.	pH	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	5.0	50	50	50	51	50	51
	8.0	50	49	50	50	50	48
1	5.0	50	50	49	50	49	49
	8.0	50	49	38	20	13	3.4
3	5.0	51	50	49	50	48	46
	8.0	51	50	5.7	2.3	0.97	0.50
6	5.0	54	52	52	50	50	50
	8.0	54	53	51	50	47	46

[a] Stored in 10-ml. vials in the dark at room temperature.

TABLE V.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AND FOLIC ACID AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	50	50	50	49	50	50
1	50	50	41	40	10	10
2	51	51	44	44	26	27
3	51	50	45	38	8.7	4.9
4	51	50	44	38	21	12
5	53	52	46	45	44	2.7
6	54	53	48	48	41	3.8

[a] Stored in 10-ml. vials in the dark at room temperature.

TABLE VI.—STABILITY OF FOLIC ACID IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AND FOLIC ACID AT pH 6.5[a]

Sample No.	Mg. of Folic Acid per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	5.0	5.0	4.7	3.9	3.5	3.4
1	5.0	5.0	4.7	4.1	3.5	3.4
2	5.0	5.0	4.3	3.9	3.5	3.5
3	5.0	5.0	4.6	3.9	3.6	3.5
4	5.0	5.0	4.4	3.6	2.8	2.8
5	5.0	5.0	3.7	2.9	2.8	2.8
6	5.0	5.0	4.3	3.3	3.2	3.2

[a] Stored in 10-ml. vials in the dark at room temperature.

potency was apparent, however, in all samples at the end of 3 months. There was no further loss between 3 and 6 months except in samples 3 and 4, but there was marked destruction of vitamin B_{12} in the first four samples at the end of 12 months. Greater destruction occurred in some solutions when the vials were inverted. This was most noticeable in samples 5 and 6.

Folic acid was stable in all solutions in upright vials during the first month (Table VI). A loss in potency was apparent at the end of 3 months and continued throughout further storage. Except for samples 4 and 5, the loss of folic acid activity observed in the liver-vitamin solutions was the same as in the control solution. Inversion of the vials had no noticeable effect on the folic acid activity of any of the solutions.

DISCUSSION

In the examination of the effect of liver solutions upon stability of cyanocobalamin, no single commercial product has been used. It was convenient to utilize combinations of commercial products available as samples in this laboratory, combined arbitrarily to provide a sufficient amount of test material for the six stock solutions that were studied. Therefore, the data presented here do not relate specifically to any particular manufacturer.

The factors, aside from composition, considered of possible importance in this study were the amount of air space, the pH of the solution, time of storage, and the possible effect of the rubber closures. It is apparent that a variation in the amount of oxygen contained in the vial was not directly related to the degree of deterioration of vitamin B_{12}. On the other hand, stability was markedly affected by pH of the solution. It was clear also that in some instances, particularly in the presence of folic acid, the rubber closure did affect the rate and degree of deterioration.

The most important factor, however, is some characteristic of the liver products used. There is no basis in results of this study for suggesting the nature of the variant in the liver products that is related to cyanocobalamin deterioration. Without knowledge of the method of manufacture or the source material, it is not possible to do other than point to the liver preparation as a principal source of the factor responsible for the decreased stability of vitamin B_{12} in these preparations.

The study reported here has been carried out under well-controlled conditions and demonstrates, beyond question, the magnitude of the losses of vitamin B_{12}, and in some cases folic acid, that can be expected when liver preparations that are incompatible with these compounds are used. It is emphasized again, as Gakenheimer (16) stated in 1952, that liver solutions to be used in injectable preparations with added cyanocobalamin require pretesting for compatibility.

SUMMARY

1. A study was conducted for a period of 12 months to obtain further information on the stability of cyanocobalamin when added to liver injectable preparations, with and without the addition of folic acid.

2. Loss of vitamin B_{12} potency in some of the liver solutions occurred during 3 months storage and continued in these preparations during further storage.

3. In the solutions tested, potency decreased when the pH was increased from 5.0 to 6.5 and from 6.5 to 8.0.

4. The loss of vitamin B_{12} was greater in some preparations when folic acid was added.

5. There was further loss of vitamin B_{12} in some samples when the solutions in the vials were in contact with the rubber stopper.

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Stability of Anionic Complexes of Some Barbituric Acid Derivatives and Silver

By JAMES P. LEYDA† and LOYD E. HARRIS

Stability constants for the anionic silver complexes for a series of barbituric acid derivatives were determined potentiometrically. The titration efficiency or per cent recovery was shown to be acceptable for reproducible results and accurate calculation of the stability constants. The effect of silver ion on the stability of barbital was determined. The presence of silver ion was shown not to interfere with the assay of barbital in alkaline solution. A structure for the anionic silver barbiturate complex was proposed.

NUMEROUS procedures for the qualitative and quantitative analysis of barbituric acid derivatives are found in the literature which employ metal ion interactions. These methods include complexation by silver, cobaltous, cupric, and mercuric ions.

In 1934, Budde (1) first employed silver nitrate as a titrant for barbiturates in alkaline solution. The appearance of a permanent turbidity was used as the end point. This turbidity is caused by the formation of the insoluble disilver barbiturate.

Danielsson (2) modified the original Budde method by replacing the sodium carbonate solution with a solution of potassium metaborate. Potassium chromate was used as the indicator. At the end point in this method one mole of silver ion is equivalent to two moles of 5,5-disubstituted barbituric acid.

Potentiometric titration procedures have recently been reported employing silver complexation in barbiturate analysis. Gautier, *et al.* (3), titrated the protonated pyridine which forms after the interaction of silver ion with barbiturate in anhydrous pyridine. Perelman (4, 5) and Poethke and Furst (6) have potentiometrically titrated barbiturates with silver nitrate in a sodium carbonate solution.

The aims of this investigation were to determine the stability of the monosilver barbiturate anionic complexes of a series of nine barbiturates and the effect of the silver complex on the stability of barbital.

EXPERIMENTAL

Reagents.—The barbiturates used in this investigation were recrystallized from ethanol-water mixtures with the exception of butethal which was recrystallized from an ether-petroleum ether (b.p. 30–60°) mixture. Sodium carbonate monohydrate and potassium nitrate were of analytical reagent grade and were not further purified.

The silver nitrate solution was obtained from the Ohio State University reagent laboratory and was standardized against ammonium thiocyanate using acidified ferric alum as the indicator.

Apparatus.—A 5-ml. buret graduated in 0.01-ml. increments; magnetic stirrer; Rubicon model B and a Leeds-Northrup potentiometer with a General Electric galvanometer as the null instrument; silver electrode, prepared from 24-gauge silver wire; calomel electrode, containing a saturated potassium chloride solution; Beckman DU spectrophotometer, and Beckman pH meter, model GS.

Potentiometric Procedure.—An accurately weighed sample (between 15 and 80 mg./100 ml.) was dissolved in a sufficient amount of an alkaline carbonate solution (pH 11.0, 0.16 *M* sodium carbonate and 0.1 *M* potassium nitrate) to make 100 ml. A 25-ml. aliquot of the sample solution was placed in a 180-ml. titration vessel and titrated with the standardized 0.1 *N* silver nitrate solution. After each addition of titrant the E.M.F. was measured using a silver electrode as indicator electrode and a calomel reference electrode. The titration was continued until the permanent turbidity appeared, which was the visual end point described by Budde. A plot of Δ mv./Δ ml. *vs.* ml. of titrant determined the end point used in the calculation of titration efficiency and stability constants. At the end point, the average Δ mv./Δ ml. was about 300. A typical titration curve for barbital appears in Fig. 1.

Calculation of Stability Constants.—The reactions which lead to the formation of the monosilver barbiturate anionic complex in alkaline solution is shown by the following sequence

$$\mathrm{BarbH} + \mathrm{Na_2CO_3} \rightleftarrows \mathrm{Barb^-\ Na^+} + \mathrm{NaHCO_3}$$

$$\mathrm{Barb^-\ Na^+} + \mathrm{AgNO_3} + \mathrm{Na_2CO_3} \rightleftarrows \mathrm{BarbAg^-\ Na^+} + \mathrm{NaNO_3} + \mathrm{NaHCO_3}$$

The equilibrium constant (instability constant) which defines the reaction is

$$K_{ins} = \frac{[\mathrm{Barb^-}][\mathrm{Ag^+}]}{[\mathrm{BarbAg^-}]}$$

The stability constant of this anionic complex is the reciprocal of the K_{ins}

$$K_s = \frac{[\mathrm{BarbAg^-}]}{[\mathrm{Barb^-}][\mathrm{Ag^+}]}$$

Kolthoff (7) has given an equation for the calcula-

Received August 28, 1961, from the Ohio State University, College of Pharmacy, Columbus 10.
Accepted for publication October 14, 1961.
† Predoctoral Fellowship (MF-11,278) from the National Institute of Mental Health, United States Public Health Service, Bethesda, Md.

TABLE I.—POTENTIOMETRIC TITRATION DATA

Compound	Samples	% Recovery	$E_{1/2}$ mv. Av.	$K_s \times 10^{-6}$	pKa (9)
Barbital	18	101.1 ± 1.7	170.7	3.46 ± 0.48	8.06
Phenobarbital	18	98.5 ± 1.5	163.4	5.03 ± 1.03	7.54
Diallylbarbituric acid	18	98.9 ± 1.3	166.6	4.60 ± 0.55	7.88
Aprobarbital	12	100.8 ● 1.9	169.0	3.81 ± 0.48	8.09
Vinbarbital	18	100.7 ± 1.7	165.0	3.85 ± 0.74	7.72
Probarbital	12	100.3 ± 2.4	170.7	3.39 ± 0.75	8.17
Butethal	12	98.2 ± 3.4	164.8	4.09 ± 1.04	8.10
Butabarbital	12	100.5 ± 2.3	171.5	3.46 ± 0.59	8.16
Amobarbital	6	98.6 ± 3.1	165.6	3.96 ± 0.20	8.02

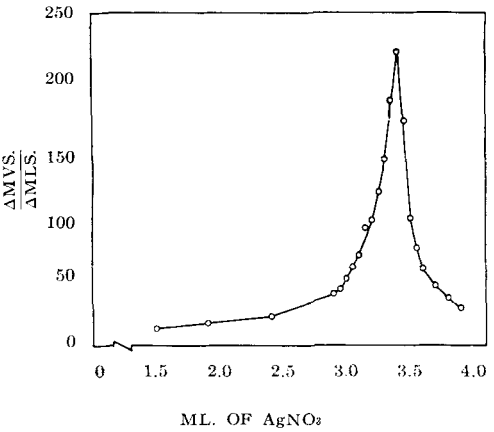


Fig. 1.—A typical first derivative titration curve for barbital titrated with silver nitrate.

tion of free silver ion concentration by measuring the potential using a silver electrode and calomel electrode system

$$p\mathrm{Ag} = \frac{0.7991 - E_s - E_c}{0.0591}$$

Where E_c is the E.M.F. of the calomel electrode which is 0.2444 v. when the electrode contains a saturated solution of potassium chloride at 25° (8). E_s is the measured E.M.F. of the system.

The concentration of anionic complex and free barbiturate was determined by the procedure described by Perelman (4, 5). Molar concentration of the free barbiturate was calculated by the equation

$$C_{\mathrm{molar}} = \frac{(c - a) \cdot N}{a + b}$$

The molar concentration of the anionic complex was calculated in a similar manner, by the equation

$$C_{\mathrm{molar}} = \frac{a \cdot N}{a + b}$$

Where a is the number of milliliters of titrant added; N is the normality of titrant (silver nitrate); b designates the number of milliliters in the original sample solution; and c is the number of milliliters of titrant at the end point, determined by the maximum Δ mv./Δ ml. In all calculations, molar concentrations were used, since the silver ion concentration was so low it was assumed that the activity coefficients were unity.

Results.—The data presented in Table I were obtained from the potentiometric titration procedure. The per cent recovery (titration efficiency) and stability constant data are the average values from a series of determinations plus the standard deviations from the mean.

Perelman (4, 5) calculated the instability constants for diallylbarbituric acid, $K_{\mathrm{ins}} = 1.1 \times 10^{-7}$ ($K_s = 9.1 \times 10^6$) and barbital, $K_{\mathrm{ins}} = 4.7 \times 10^{-8}$ ($K_s = 2.1 \times 10^7$). Poethke and Furst (6) have reported the instability constants for barbital and phenobarbital respectively, $K_{\mathrm{ins}} = 4.0 \times 10^{-7}$ ($K_s = 2.6 \times 10^6$) and 2.7×10^{-7} ($K_s = 3.7 \times 10^6$). The data presented in Table I agree with the limited number of values given by Poethke and Furst.

A general trend is noted between the stability constants of these anionic complexes and the pKa values of the parent acids. Due to the relatively narrow range which the pKa values span for the barbituric acids, it would be expected that the stability constants would be of similar magnitude. The stability of the complexes are of the same magnitude and slightly increase with the decrease in the pKa of the parent acid.

Effect of Silver Ion on the Molar Absorptivity of Barbital.—Danielsson (2) reported that the molar absorptivity of the silver-barbital anion at the pH of 10.9 appears to be 18,820 when determined at 238 mμ. Previously, the molar absorptivity of barbital, at 239 mμ and the pH 10.9, has been shown to be 10.2×10^3 (9). In order to assay barbital in the presence of silver ion and silver-barbital complex, the absorbances of these various species were determined.

The absorbances of 3.89×10^{-5} M solutions of barbital (total) were measured by a Beckman DU spectrophotometer at 239 mμ. The solutions contained varying amounts of silver ions and were buffered at pH 10.9. The data are presented in Table II.

The average molar absorptivity of the complex is 10.10×10^3, which correlated within 99.0% of the

TABLE II.—EFFECT OF SILVER ION ON THE ABSORBANCE OF BARBITAL

Sample	Barbital, $M \times 10^5$	Silver Complex, $M \times 10^5$	Free Barbital, $M \times 10^5$	A_{obs}
1	3.89	0	3.89	0.398
2	3.89	0	3.89	0.397
3	3.89	0.841	3.05	0.400
4	3.89	0.841	3.05	0.395
5	3.89	1.680	2.21	0.394
6	3.89	1.680	2.21	0.395
7	3.89	2.520	1.37	0.395
8	3.89	2.520	1.37	0.394
9	3.89	3.360	0.53	0.398
10	3.89	3.360	0.53	0.388

literature value (9) for the molar absorptivity of barbital. Thus, it appears that the spectrophotometric assay is efficient for determining the total amount of barbital in the system.

Effect of Silver Anionic Complex on the Stability of Barbital.—The decomposition of aliphatic substituted ureides in alkali has been shown to be retarded when intermolecular hydrogen bonding is present. Werner (10) described this as chelate formation. Since barbiturates are cyclic ureides which hydrolyze in alkaline solution, silver ions are believed to form a silver-barbiturate anionic complex which resists hydrolysis.

A 0.2 *M* solution of sodium carbonate (pH 11.0) was employed as the alkaline medium. Barbital solutions were prepared of approximately 4.0×10^{-3} *M* and varying amounts of silver nitrate. Samples were sealed in 2-ml. Neutraglas ampuls and placed in an oil bath at 42.5°. At various time intervals, samples were removed from the constant temperature bath and immediately frozen in a chloroform-dry ice mixture. The samples were assayed spectrophotometrically as previously described.

The degradation rate (apparent) is defined by the following equation

$$k_{app} = N_{barb}\, k_{barb} + N_{barbAg}\, k_{barbAg}$$

Where N_{barb} is the mole fraction of free barbital, N_{barbAg} the mole fraction of silver-barbital complex (silver mole fraction), k_{barb} the pseudo first-order degradation rate of free barbital, and k_{barbAg} the pseudo first-order degradation rate of the complex.

The results are presented in Table III, and Fig. 2 illustrates the relationship between the pseudo first-order reaction rate and the concentration of anionic silver-barbital complex. The comparison of the reaction rate constants between the control samples and the samples containing known amounts of silver ion concentration agrees within 97.7% of the calculated rate of hydrolysis (assuming that only the free barbital degrades). Calculated data (solid line) had a slope -5.43×10^{-5} and the experimental data (broken line) had a slope -5.56×10^{-5}, determined by the method of least squares.

TABLE III.—EFFECT OF SILVER ION ON THE DEGRADATION OF BARBITAL

Sample	Mole Fraction Silver Complex	$k_{app} \times 10^3$ hr.$^{-1}$	$k_{barb} \times 10^3$ hr.$^{-1}$ (calcd.)
1	0	5.42	5.42
2	0	5.49	5.49
3	0.268	3.78	3.97
4	0.576	1.93	2.31
5	0.836	0.82	0.89

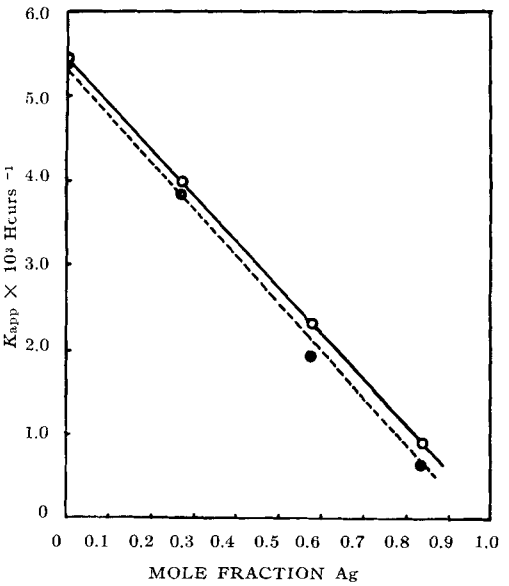
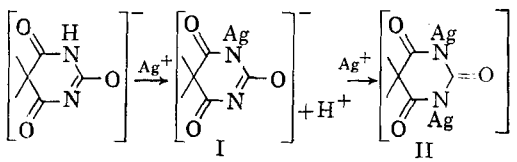


Fig. 2.—The relationship between pseudo-first order degradation rate and the mole fraction of silver. ——O—— represents the calculated data and - - ● - - the experimental data.

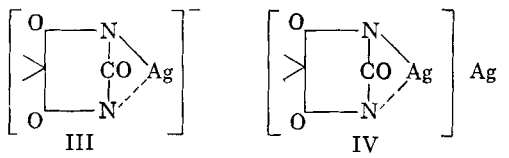
DISCUSSION AND RESULTS

Perelman (4) has described the reaction sequence for the formation of the silver complexes of barbiturates. In an alkaline solution, barbiturates react with silver ions in a step-wise fashion which is shown by the following formulas

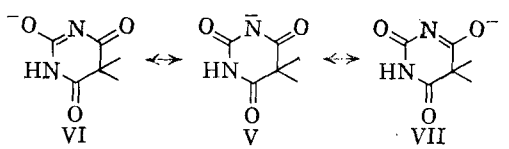


Gautier, *et al.* (3), have followed the released protons of these reactions in pyridine. They have shown that titration of the free barbituric acid liberated two protons and that one proton is liberated when the sodium salts are employed.

Poethke and Furst (6) represent the following formulas as the anionic complex (III) and the water-insoluble disilver barbiturate (IV) as chelate compounds



Goyan, *et al.* (11), have written the resonance forms of the anionic species in the following manner

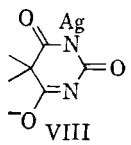


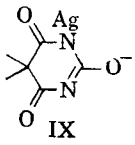
Spectral data have supported the view that species V is not the resonance form which is attacked by the silver ion. The enol forms which are present in alkaline solution are the species responsible for the increase in absorbance and are represented by structures VI and VII. We have shown that the silver-barbital anionic complex does not appear to alter the absorptivity of the barbiturate. Thus, the

silver ion does not change the electron distribution in the enolized form.

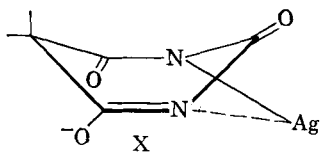
Goyan, *et al.* (11), have suggested that barbital degradation in ammonia buffer is similar to ammonolysis of an ester and that hydrolysis of the ionized species would probably be at the 4 (or 6) position. By virtue of infrared spectra of the *p*-nitrobenzyl derivatives of a series of barbiturates, Chatten and Levi (12) have proposed that the mechanism of the reaction involves enolization of the carbonyl at position 4 (or 6).

The silver-barbiturate anionic complex has been shown to be stable in alkaline solution. The silver ion, when attacking the barbiturate anion, liberates a proton, thus the nitrogen atom which is not involved in the enolization appears to be the position of the silver ion, as shown by structures VIII and IX


VIII


IX

These formulas do not appear to explain the stability of the complex to alkaline hydrolysis, especially if the 4 (or 6) carbonyl group is the position vulnerable to hydroxyl ion attack.


X

Barbiturates which have substituents on a nitrogen atom, i.e., a methyl group, are known to hydrolyze in the same way as the unsubstituted compounds. The mono silver anionic complexes are believed to be stabilized by the formation of a chelate structure involving both the 1 and 3 nitrogen atoms. This compound was previously described by Poethke and Furst (6) and is represented by structure X.

SUMMARY

1. Stability constants for the anionic silver complexes for a series of nine barbituric acid derivatives were determined potentiometrically.

2. The per cent recovery or titration efficiency was determined.

3. The molar absorptivity of the silver anionic complex of barbital was shown to be the same as that of barbital in alkaline solution.

4. The effect of silver ion on the stability of barbital in a sodium carbonate solution was described.

5. A structure for the anionic silver complex was proposed.

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Synthesis of Some Derivatives of Sorbic Acid and Their Antifungal Properties

By ROBERT W. GOETTSCH†, AUGUST G. DANTI, and DALE H. CRONK

Seven amides and three amine salts of sorbic acid were prepared and screened for their antifungal activity against four pathogenic fungi. Of the 10 compounds tested, N-(2-methylpiperidyl)-sorbamide, *n*-hexylsorbamide, cyclohexylamine sorbate, phenylethylamine sorbate, and *n*-hexylamine sorbate exhibited antifungal activity.

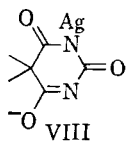
THERE ARE many types of chemical compounds that have been studied for their potential antifungal activity. Among the diverse chemical types of antifungal agents investigated are the fatty acids, their salts, and derivatives. Hoffman, Schweitzer, and Dalby (1) have demonstrated that fatty acids and their salts are fungistatic for certain nonpathogenic fungi. Keeney and Broyles (2) reported that sodium propionate appeared effective in the treatment of certain superficial fungus infections. Keeney, Ajello, and Lankford (3) investigated the fungi-

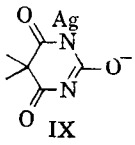
Received October 30, 1961, from the School of Pharmacy, Northeast Louisiana State College, Monroe.
Accepted for publication December 7, 1961.
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silver ion does not change the electron distribution in the enolized form.

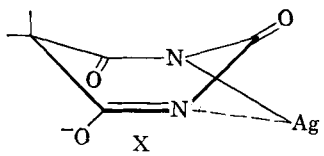
Goyan, *et al.* (11), have suggested that barbital degradation in ammonia buffer is similar to ammonolysis of an ester and that hydrolysis of the ionized species would probably be at the 4 (or 6) position. By virtue of infrared spectra of the *p*-nitrobenzyl derivatives of a series of barbiturates, Chatten and Levi (12) have proposed that the mechanism of the reaction involves enolization of the carbonyl at position 4 (or 6).

The silver-barbiturate anionic complex has been shown to be stable in alkaline solution. The silver ion, when attacking the barbiturate anion, liberates a proton, thus the nitrogen atom which is not involved in the enolization appears to be the position of the silver ion, as shown by structures VIII and IX


VIII


IX

These formulas do not appear to explain the stability of the complex to alkaline hydrolysis, especially if the 4 (or 6) carbonyl group is the position vulnerable to hydroxyl ion attack.


X

Barbiturates which have substituents on a nitrogen atom, i.e., a methyl group, are known to hydrolyze in the same way as the unsubstituted compounds. The mono silver anionic complexes are believed to be stabilized by the formation of a chelate structure involving both the 1 and 3 nitrogen atoms. This compound was previously described by Poethke and Furst (6) and is represented by structure X.

SUMMARY

1. Stability constants for the anionic silver complexes for a series of nine barbituric acid derivatives were determined potentiometrically.

2. The per cent recovery or titration efficiency was determined.

3. The molar absorptivity of the silver anionic complex of barbital was shown to be the same as that of barbital in alkaline solution.

4. The effect of silver ion on the stability of barbital in a sodium carbonate solution was described.

5. A structure for the anionic silver complex was proposed.

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By ROBERT W. GOETTSCH†, AUGUST G. DANTI, and DALE H. CRONK

Seven amides and three amine salts of sorbic acid were prepared and screened for their antifungal activity against four pathogenic fungi. Of the 10 compounds tested, N-(2-methylpiperidyl)-sorbamide, *n*-hexylsorbamide, cyclohexylamine sorbate, phenylethylamine sorbate, and *n*-hexylamine sorbate exhibited antifungal activity.

THERE ARE many types of chemical compounds that have been studied for their potential antifungal activity. Among the diverse chemical types of antifungal agents investigated are the fatty acids, their salts, and derivatives. Hoffman, Schweitzer, and Dalby (1) have demonstrated that fatty acids and their salts are fungistatic for certain nonpathogenic fungi. Keeney and Broyles (2) reported that sodium propionate appeared effective in the treatment of certain superficial fungus infections. Keeney, Ajello, and Lankford (3) investigated the fungi-

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static and fungicidal activity of fatty acid salts and found that fungicidal activity was acquired as the fatty acid chain was lengthened. Gooding (4) and Geigy (5) reported that certain α,β-unsaturated fatty acids and their derivatives, respectively, possessed fungistatic activity. Peck and Rosenfeld (6) found that olefinic acids possessed antifungal activity to a greater degree than their aliphatic homologs.

Sorbic acid, which is intermediate in the fatty acid series in respect to carbon chain length and is unsaturated at the α,β and γ,δ-positions, and its derivatives have been studied for their antifungal activity. Puls, Lindgren, and Cosgrove (7) reported that sorbic acid was superior to benzoic acid as a fungistatic agent. Recently some derivatives of sorbic acid were reported by Cronk, Zopf, and Jones (8) which have shown the potentialities of sorbic acid derivatives as antifungal agents. This investigation was undertaken to study the effects of some sorbic acid derivatives on certain pathogenic fungi which produce dermatoses in man.

EXPERIMENTAL PART I

Sorbyl Chloride.—To 160 Gm. (1.43 moles) of sorbic acid[1] in a 1-L. round-bottom flask was gradually added 328 Gm. (2.75 moles) of thionyl chloride. After addition of the thionyl chloride, the mixture was stirred for one and one-half hours and then heated to 80° for one-half hour. The product was purified by vacuum distillation using potassium hydroxide and a mixture of dry ice and acetone as traps. The fraction distilling at 95–99° (20 mm. Hg) was collected as a nearly colorless liquid. The yield was 135 Gm. or 72% of the theoretical.

Method I.—The substituted amides of sorbic acid were prepared by reaction of sorbyl chloride with the appropriate amine as illustrated by the following process.

N-Benzylsorbamide.—In a 500-ml. round-bottom flask was placed 6.5 Gm. (0.05 mole) of sorbyl chloride dissolved in 200 ml. of ether. A solution of 10.7 Gm. (0.1 mole) of benzylamine in 50 ml. of ether was added, dropwise. An immediate reaction took place with formation of a precipitate of the amine hydrochloride. The mixture was stirred for 30 minutes at room temperature; the resulting mixture was treated with water and the ether layer separated. The ether layer was washed with 5% hydrochloric acid, 4% sodium carbonate, and water. Evaporation of the ether layer gave the sorbamide which was purified by recrystallization from ethanol. The yield was 8.5 Gm. (84% of theoretical) of white crystals which melted at 129–131°.

Method II.—The amine salts of sorbic acid were prepared by reaction of sorbic acid with the appropriate amine as illustrated by the following process.

N-Cyclohexylamine Sorbate.—In a 500-ml. round-bottom flask was placed 5.6 Gm. (0.05 mole) of sorbic acid dissolved in 200 ml. of ether. A solution of 6.94 Gm. (0.07 mole) of cyclohexylamine, in 50 ml. of ether was added, dropwise. The mixture was stirred for one and one-half hours at room temperature and the resulting precipitate filtered and dried. The salt was purified by recrystallization from ethyl acetate. The yield was 9.6 Gm. (91% of theoretical) of white crystals which melted at 150–153°.

The recrystallization solvents, yields, melting points, and analyses are given in Table I.

EXPERIMENTAL PART II

The antifungal activity of the compounds prepared in this investigation was determined by the paper disk technique. The fungi used were: *Microsporum canis* (ATCC10214), *Microsporum gypseum* (ATCC10215), *Microsporum audouini* (ATCC10216), and *Trichophyton rubrum* (ATCC-10218). Aqueous suspensions of spores of the test organisms were prepared from 14-day agar slants which had been incubated at 28 ± 2°. The suspensions were prepared by aseptically transferring pure colony growths from the slants to a sterile 4-oz. wide-mouth glass bottle. One-half-inch sections of glass rod and 40 ml. of sterile distilled water were added to the bottle, then agitated vigorously to pulverize the colony growth into a homogeneous suspension.

The culture plates were prepared using Difco Sabouraud's dextrose agar dehydrated, 65 Gm./L., pH 5.6. Exactly 20 ml. of the dissolved agar was pipetted into test tubes and autoclaved for 15 minutes at 15 pounds pressure (121°). The media from each test tube was poured into sterile, flat bottom Petri dishes (15 × 100 mm.) and allowed to solidify. The plates were then inoculated with 0.5 ml. of the spore suspensions.

The test compounds, with the exception of cyclohexylamine sorbate, phenylethylamine sorbate, and *n*-hexylamine sorbate were dissolved in acetone in 5% w/v concentrations. The above salts were dissolved in water in the same concentration.

The testing procedure consisted of immersing the sterile paper disks[2] in the test solution of each compound and draining off the excess liquid by touching the disk to the wall of the container. One disk was placed in the center of each culture plate, running triplicate plates for each compound. The plates were then incubated at 28 ± 2° for 14 days. The zones of inhibition were recorded at the end of 7 and 14 days. The zones of inhibition were measured as the minimum distance from the periphery of the paper disk to the edge of the colony growth. The *in vitro* results of the test compounds and controls employed are given in Table II.

DISCUSSION

During the course of this investigation other derivatives were prepared in addition to those reported in Table I, namely N-piperidylsorbamide, N-(*n*-butyl)-sorbamide, N-(*sec*-butyl)-sorbamide, N-(2-aminopyridyl)-sorbamide, and 1-methyl-4-sorbyl-

[1] The sorbic acid used in this investigation was supplied through the courtesy of Union Carbide Chemicals Co., Division of Union Carbide Corp., New York 17, N. Y.

[2] Difco Bacto disks, sterile blanks, 1/2 inch (13 mm.).

TABLE I.—AMIDES AND AMINE SALTS OF SORBIC ACID

No.	Amine Component	Recrystallization Solvent	Yield, %	M.P., B.P.,[a] °C.	Formula	Nitrogen—Calcd.	Nitrogen—Found
1	Benzylamine	Ethanol	84	129–131	$C_{13}H_{15}NO$	6.95	6.72
2	Cyclohexylamine	Ethanol	88	161–162	$C_{12}H_{19}NO$	7.25	7.18
3	Phenylethylamine	Isopropanol	83	115–116	$C_{14}H_{17}NO$	6.51	6.45
4	2-Methylpiperidine	None	76	185 (20 mm.)	$C_{12}H_{19}NO$	7.25	6.97
5	*n*-Hexylamine	Ethyl ether-petroleum ether	77	74–75	$C_{12}H_{21}NO$	7.17	6.86
6	*o*-Toluidine[b]	Ethanol	86	171–173	$C_{13}H_{15}NO$	6.95	6.95
7	*p*-Phenetidine	Ethanol	81	170–172	$C_{14}H_{17}NO_2$	6.06	6.00
8	Cyclohexylamine[c]	Ethyl acetate	91	150–153	$C_{12}H_{21}NO_2$	6.64	6.80
9	Phenylethylamine[c]	Ethyl acetate	77	100–102	$C_{14}H_{19}NO_2$	6.00	6.01
10	*n*-Hexylamine[c]	Ethyl acetate	80	99–101	$C_{12}H_{23}NO_2$	6.57	6.55

[a] All melting points are uncorrected. [b] Previously reported m.p. 173° [see (11)]. [c] Amine salts of sorbic acid.

TABLE II.—ZONES OF INHIBITION, COMPOUNDS AT 5% w/v CONCENTRATIONS[a]

Compound No.	*M. canis* Days 7	*M. canis* Days 14	*M. gypseum* Days 7	*M. gypseum* Days 14	*M. audouini* Days 7	*M. audouini* Days 14	*T. rubrum* Days 7	*T. rubrum* Days 14
1	0	0	0	0	0	0	0	0
2	1	0	0	0	3	1	0	0
3	2	0	0	0	8	1	1.5	0
4	16	7	19	13.5	20	16.5	16.5	9
5	3	2.5	3	1.5	9	3	5	1
6	0	0	0	0	1	0	0	0
7	0	0	0	0	1	0	0	0
8	16	0	0	0	25.5	12	10.5	0
9	12.5	0	0	0	24.5	17	11	0
10	10.5	0	2	0	26	13	10.5	0
Sorbic acid	16	0	2.5	0	31.5	19.5	15.5	0
Acetone	0	0	0	0	0	0	0	0

[a] Zones, minimum distance from periphery of disk to edge of colony growth, average of three measurements. Measurements given in millimeters.

piperazine. Other salts prepared were benzylamine sorbate, piperidine sorbate, 1-methylpiperazine sorbate, 2-methylpiperidine sorbate, *n*-butylamine sorbate, *sec*-butylamine sorbate, and 2-aminopyridine sorbate.

It was found that many of the sorbamides and amine salts were unstable with respect to light and/or room temperature and decomposed with the formation of light tan to dark brown solids or resinous products. The unstable nature of amine salts and derivatives is well known. A technical bulletin from Union Carbide Corp. (9), in referring to amine soaps (salts), states that the color becomes somewhat darker on standing and that this color formation varies with both the amine component and the oxidizing tendencies of the fatty acid being used, but that the color change does not impair the effectiveness of the compounds. According to a treatise on sorbic acid (2,4-hexadienoic acid) (10), the acid, its esters, and salts are subject to oxidative reactions at the olefinic bonds and that autoxidation results in peroxide formation followed by a degradative and polymerization process. This ease of oxidation apparently accounts for some of the decomposition noted in the compounds prepared during this study.

In preparing the solutions for antifungal testing it was found that compounds No. 2, 3, 6, and 7 (Table I) had a maximum solubility of less than 5% w/v in acetone. This factor may have contributed to the little or no antifungal properties shown for these compounds (Table II). The fact that some solutions were made with acetone and some with water did not exhibit any discernible degree of difference in diffusion rates when the paper disks were placed on the agar media.

It should be noted that although the readings were taken on the 7th and 14th days, that in the case of *M. gypseum*, compounds No. 8, 9, and 10, (Table I), as well as the control, sorbic acid, were effective up to 5 days. After this time growth resumed, until at the end of 2 weeks, no zones of inhibition were perceived.

Compounds No. 4, 5, 8, 9, and 10 (Table I) showed the greatest degree of antifungal activity against all pathogens tested (Table II). With the exception of the test with *M. audouini*, N-(2-methylpiperidyl)-sorbamide appeared to be a more effective antifungal agent than was sorbic acid after 2 weeks of culture growth.

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X-ray and Crystallographic Applications in Pharmaceutical Research I

Trace Element Determinations by X-ray Emission

By JOHN W. SHELL†

A standard procedure generally applicable on a routine basis to the determination of any heavy element in a solid organic matrix is described, using an improved method for the determination of selenium in steroids as an example. The general method is applicable for both high and low concentrations including, as is illustrated, trace quantities. An exception to the general application occurs with mercury, due to interferences from primary radiation, and an alternate procedure is described for this element. Extension of the method to lighter elements is illustrated by procedures for the determination of chromium and manganese, and a critical comparison of alternate procedures for these two elements is made. A statistical study of common errors showing optimum conditions for lighter elements is also presented.

X-RAY and crystallographic methods are well known in some fields, but wide usage in the organic, particularly the pharmaceutical field, has not yet occurred. These methods as applied to a wide variety of pharmaceutical problems are valuable and extremely versatile.

A series of papers, of which this is the first, will describe the use of these methods as they have been found to apply to typical pharmaceutical problems. Subsequent papers of the series will be concerned with modified molecular weight determination procedures, quantitative and qualitative diffraction analyses, crystal habit quantitation, low angle scattering applications, and crystal phase transitions.

X-ray emission analysis (also called X-ray fluorescence analysis) has expanded rapidly in recent years. Two excellent books on the method (1, 2) have recently been published, and cover the fundamentals. A review in 1960 (3) listed 223 papers on applications of X-ray absorption and emission, but very few of these were related to organic systems.

Several advantages of the X-ray emission method make it especially adaptable for pharmaceutical problems. Due to the low atomic number of carbon, hydrogen, and oxygen constituting organic systems, matrix component interferences, encountered in many X-ray emission applications, are absent. Moreover, the method is applicable directly on samples, either liquid or solid, and usually without need for separations, permitting assays on final product formulations. Further advantages are speed, specificity, the nondestructive nature of the method, and the inherent simplicity of X-ray spectra. The nonapplicability to low atomic numbered elements is a disadvantage when considering X-ray emission as a general method, but is a decided advantage when the method is used for the determination of heavy elements in a light (organic) matrix.

A method for the simultaneous determination of traces of selenium and mercury in pharmaceuticals was previously reported from these laboratories (4). This method has been greatly improved, particularly in speed and sample handling conveniences, and developed into a standard procedure generally applicable on a routine basis to the determination of any heavy element in a solid organic matrix. This paper describes the general method as applied to trace quantities of elements, such as residual toxic catalysts, and treats special problems encountered in determinations of low concentrations. The same general method and procedures as described are readily applied to the determination of elements at higher concentrations, making it ideally useful for the determination of mineral ingredients in multivitamin products and other preparations.

DISCUSSION

Principle of the Method.—Several processes can be expected to occur when matter is bombarded by intense radiant energy. One of these is a quantum process wherein atomic electrons are displaced, and the vacancies caused are then filled by electrons from an outer shell. Such a process must be accompanied by a release of energy, and it is significant that this energy is in the form of X-rays. As there are several energy levels associated with the various electron shells, quantum theory predicts a number of

Received October 26, 1961, from the Pharmacy Research Section, Product Research and Development Unit, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication November 16, 1961.
Presented to the First Annual Conference on Pharmaceutical Analysis, Land O'Lakes, Wis., September 1961.
It is a pleasure to acknowledge the technical assistance of Dr. J. I. Northam, The Upjohn Co., who provided aid in the statistical analyses, and Mr. Karl Zipple, also of The Upjohn Co., who prepared some of the standard samples and collected a portion of the counting data used in this work.
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possible transitions, some more likely than others. Thus, an element when bombarded by sufficiently intense radiant energy will emit several X-ray "lines," each at a specific wavelength, characteristic of the element. This is the qualitative aspect of analysis. The intensity of any selected line is proportional to, among other factors, the number of emitting atoms. This, following independent calibration, is the quantitative aspect of emission analysis.

In practice, the radiant energy is supplied by an X-ray tube. The determination of wavelength of the emitted X-ray is accomplished by diffraction, using an analyzing crystal of known orientation and known interplanar spacing; the wavelength is then given by solution of the Bragg equation. The intensity of the diffracted beam is measured by an electronic counter and scaler circuit. The commercially available spectrometers automatically plot this intensity against the Bragg angle, 2θ, which is readily converted to wavelength by the use of tables for a given analyzing crystal. For highly accurate quantitative work, the spectrometer is set for a given wavelength (2θ value), and the counter allowed to accumulate either a fixed number of counts, or to count for a fixed period of time, from which intensity, in counts-per-second, is determined. The procedure generally requires independent standardization, using standards of known concentration.

Sample Preparation.—In preparing samples for analysis it has been found that solid organic compounds are most conveniently handled by briquetting. In this study, 0.5-Gm. samples were compressed in a Perkin-Elmer potassium bromide die at 6000 lb. pressure, without evacuation. This produced pellets of 0.5 in. diam. and, for steroids, about $^1/_8$ in. thick. These samples do not possess "infinite thickness" with respect to the X-ray beam, but since the sample weight, diameter and pressure used are constant, the total mass irradiated remains essentially constant for wide variations in matrix density.

Instrumentation.—A General Electric XRD-5 spectrometer, equipped with a lithium fluoride analyzing crystal, was used for all measurements. Most of the experimental work was completed using a tungsten target X-ray tube (Machlett AEG-50T) operated at 50 kvp and 45 ma. The platinum target tube used for one experiment was also operated at these settings. The scintillation counter used was operated at a plateau voltage of 1.275 kv, and the flow proportional counter (G.E. No. 4SPG) was operated at 2.0 kv, except when these counters were used in connection with a reverter circuit. In instances where a reverter was used, the particular peak voltages employed are listed in the individual sections which follow. The standard mask of the XRD-5 sample drawer was replaced with an aluminum plate, milled with a recession into which the half-inch sample briquettes were placed for analysis.

EXPERIMENTAL

General Illustrative Example. The Determination of Selenium.—Through use of selenium as a catalyst in some organic syntheses, this toxic element can appear in final pharmaceutical products. The determination of selenium in the parts-per-million range is a good example to illustrate application of the general method, as selenium represents an element whose atomic number ($Z = 34$) is high enough so that it may be readily determined. Elements of higher atomic number are determined with even less difficulty.

Selenium standards were prepared by adding aqueous solutions of selenium dioxide, representing several concentrations of selenium, to beakers containing in each 0.5 Gm. steroidal material in acetone solution. After mixing, the solutions were allowed to evaporate to dryness. No heat or vacuum was used, due to the volatility of selenium. The solution step assured homogeneity of selenium dispersions, and relatively little selenium was lost on the beaker surfaces due to the relatively greater adsorbing surface presented by the precipitating steroid crystals. Mixed residues of each beaker were then quantitatively transferred to the die and compressed as described above in the section on sample preparation. The standard briquettes were placed directly into the depression milled into the sample drawer plate.

For this study the XRD-5 spectrometer was equipped with a tungsten target X-ray tube and lithium fluoride analyzing crystal. A scintillation counter fed impulses through a reverter, used to discriminate electronically against undesired wavelengths, to a simultaneously operating decade scaler and timer. The spectrometer was set for $2\theta = 31.89°$, the proper position to measure the Kα emission line of selenium. Following insertion of a high selenium-bearing sample in the sample drawer, the analyzing crystal position was carefully "tuned" to produce maximum counting rate. Adjustments of the crystal position were followed by an automatic slow scan (0.2 degrees per minute) with the recorder, which plotted counting rate *vs.* degrees 2θ, until the maximum rate occurred at exactly 31.89°. A counter voltage of 1.175 kv was found to give maximum counting rate with the reverter for selenium Kα radiation.

The detector was set to record the time required for the accumulation of 200,000 counts, and this time was measured for each of the standard selenium samples while the instrument was set for $2\theta = 31.89°$, the selenium line, and at $2\theta = 34.89°$, a convenient setting for background determination. The peak to background ratio, P/B, was computed for each standard sample.

$$P/B = \frac{\text{selenium, counts-per-second}}{\text{background, counts-per-second}} = \frac{\text{time at background for 200,000 counts}}{\text{time at selenium line for 200,000 counts}}$$

The values for P/B were plotted *vs.* concentration of selenium to give the straight line shown in Fig. 1.

Repeated determinations of the standard curve shown in Fig. 1 from the same samples have shown good reproduction of both the direction and the magnitude of the scatter of each sample value from the line. The method appears to be limited most by lack of complete homogeneity of standard samples, a fact not surprising in low part-per-million concentrations.

Typical times recorded for the required count accumulation range from 167 sec. for the 0 p.p.m.

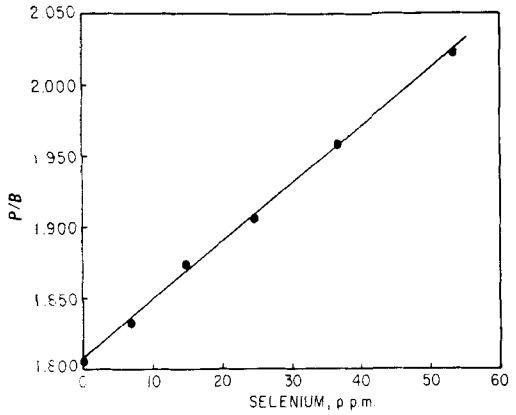


Fig. 1.—Selenium, peak to background ratio *vs.* concentration.

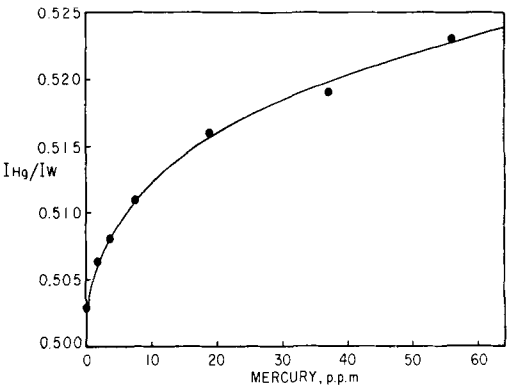


Fig. 2.—Mercury, intensity ratio *vs.* concentration.

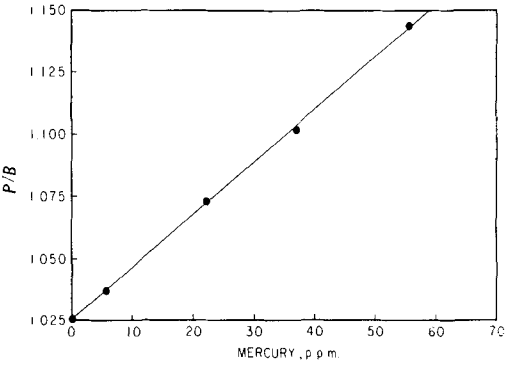


Fig. 3.—Mercury, peak to background ratio *vs.* concentration.

sample to 152.8 sec. for the 35.6 p.p.m. sample. Following standardization, the briquetted unknown samples require only a few minutes to assay.

An Instance Requiring Solution of Interference from the Primary Radiation. The Determination of Mercury.—As noted above, X-ray emission analysis of heavy elements in organic systems is virtually free from interferences from matrix components. The procedure described above for the determination of selenium can be routinely adopted for any heavy element in organic systems, with the exception of one instance. This exception occurs when the analytical emission line to be measured is coincident with, or nearly coincident with, a strong line produced by primary radiation from the X-ray tube. For instance, in the determination of mercury the analytical line of choice, Lα, is found (with a lithium fluoride crystal) at 2 θ = 35.90°. The sample scatters all radiation from the tube, and if a tungsten target tube is used a very strong line, representing tungsten Lβ, will occur at 2 θ = 37.40°. The tungsten line is so strong that even with the high resolution produced by a 0.01-in. detector slit, interference with the mercury line occurs. In fact, the mercury line is found on the steep slope of one side of the tungsten line. A determination for trace amounts of mercury under such conditions produces a standard curve with a major curvature in the low concentration region, as seen in Fig. 2. At higher concentrations of mercury, the tungsten line becomes less dominating, and the standard curve becomes more linear and therefore more useful. But for routine use at low mercury concentrations the curvature is not reproducible from day-to-day.

The best solution found to the interference problem from tungsten was replacement of the tungsten tube with one of different target material. A platinum target tube was used, and the results were a linear relationship in the standard curve for mercury, as seen in Fig. 3. This curve was found to be reproducible from day-to-day. Moreover, counting times were much less for the mercury samples with the platinum target procedure. Each point on the curve for Fig. 3 represents two countings of 40,000 counts each, and the points on the curve for Fig. 2 represent duplicate countings of one million counts each, required for comparable precision. Samples for Fig. 2 were rectilinear compressions of 200 mg. of material, and for Fig. 3 were prepared as described in the standard procedure for selenium, above.

The ratio value plotted in Fig. 2 represents the ratio of the intensity of the mercury Lα line to the intensity of a tungsten scatter line; the value P/B in Fig. 3 represents the ratio of the Lα mercury line intensity to the intensity of the background at the base of the peak. Neither of these selections for background reference appears to have an advantage over the other.

Lower Atomic Numbered Elements. A Critical Study of Alternative Methods for the Determination of Chromium and Manganese.—The applicability of X-ray emission analysis to an element in any matrix depends to a large extent upon the atomic number of the element. This is particularly true for trace analysis, even in organic matrices.

A fundamental relationship states that the reciprocal of the emission wavelength is proportional to the square of the atomic number, where the proportionality constant depends upon the emission series. Thus, lower atomic-numbered elements emit rays of longer wavelength, and these are nonpenetrating, even to air. Further, the "fluorescent yield" of an atom is much less with decreasing atomic number, due to the increasing probability of the Auger effect. A third limitation on emission from elements of low atomic number stems from the lack

of suitability of analyzing crystals with sufficiently large interplanar spacings.

From a practical standpoint, it is generally recognized that elements of atomic numbers 1 through 11 cannot be detected by present day X-ray emission equipment; that elements of atomic numbers 12 through 22 are measured only under special conditions (vacuum or helium atmosphere, and flow-proportional counter), and elements of atomic numbers 23 and higher are measured readily with no special requirements other than that listed in previous sections above for selenium and mercury.

A number of elements of pharmaceutical interest are in the lighter element region. Two such elements, of interest because of their use as catalyst in organic syntheses, are chromium ($Z = 24$), and manganese ($Z = 25$). These elements fall on a very steep portion of the "S-shaped" curve which results from a plot of fluorescent yield *vs.* atomic number (5). They also lie close to the division line for nonfeasibility of determination except under special conditions. A critical study of the effects of the special conditions on each of these two adjacent elements in this particular range was undertaken not only to observe the effects but to gain some idea of the rate of change of feasibility with increasing atomic number in this range, and under the conditions of the standard procedures employed.

Solid pellet standards for manganese and chromium were prepared by the procedure described for selenium, following evaporation of appropriate solutions of manganese dioxide and potassium dichromate. A tungsten target tube was used, and 2θ settings employed were 69.35° for the Kα line of chromium and 62.97° for the Kα line of manganese. All countings were for a fixed time of 100 sec. and no reverter circuit was employed.

Since critical comparisons were to be made in this particular study, net peak height determinations, rather than peak-to-background ratios were measured as a function of concentration, and least squares lines, as well as standard deviations of the net peak heights about the least squares lines were computed.

In this study two methods are compared. Method 1 consisted of procedures as previously described for selenium and mercury; the measurements were carried out in air, using a scintillation counter. Method 2 refers to the use of a helium atmosphere, under positive pressure, which excludes all air from the X-ray beam path, and a gas flow proportional counter in place of the scintillation counter. The gas used to flush this counter continuously was a mixture of 90% argon and 10% methane.

Graphical representation of the results of the use of methods 1 and 2 for the trace determination of both chromium and manganese is shown in Figs. 4 and 5.

The standard deviation of an estimated concentration in parts-per-million is given approximately by

$$\frac{s}{b}\sqrt{\frac{1}{m}+\frac{1}{n}}$$

where b = slope of least squares line, net peak height (c.p.s.) *vs.* concentration (p.p.m.); s = standard deviation of net peak height (c.p.s.) about the least squares line; n = number of determinations used

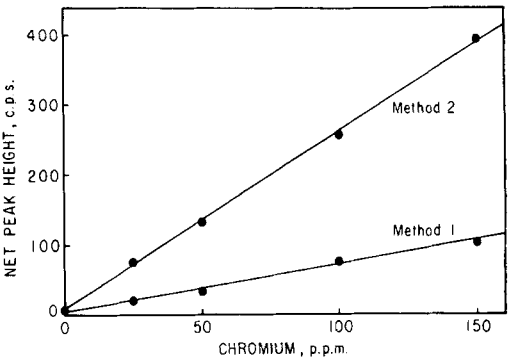


Fig. 4.—Chromium, net peak height *vs.* concentration.

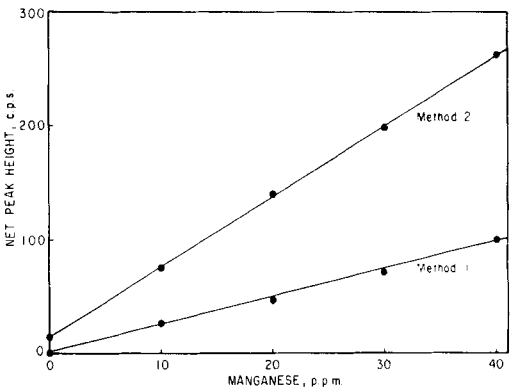


Fig. 5.—Manganese, net peak height *vs.* concentration.

in the standard curve; and m = number of determinations used in measuring the unknowns.

The value $\lambda = s/b$ is an excellent criterion for comparing alternative methods. Smaller values for λ indicate greater inherent precision of the method. Table I lists s, b, and λ values computed for methods 1 and 2 for the determination of chromium and manganese. The ratio of λ values for chromium, 6.22/1.84 = 3.38, indicates that method 2 has 3.38 times the precision of method 1 for estimating the concentration of chromium in p.p.m. Similarly, the value 1.03/0.263 = 3.92 indicates that method 2 for manganese has 3.92 times the precision of method 1. This clearly illustrates the very significant gain in precision made by use of the helium atmosphere and a flow counter for elements in this atomic number range. The λ values also show the rapid gain in feasibility as the atomic number of the element increases slightly in this range.

TABLE I.—STATISTICAL COMPARISON OF METHODS 1 AND 2 FOR THE DETERMINATION OF TRACE AMOUNTS OF CHROMIUM AND MANGANESE

	Method	s	b	λ
Chromium	1	4.216	0.67762	6.22
	2	4.685	2.5475	1.84
Manganese	1	2.544	2.470	1.03
	2	1.628	6.190	0.263

TABLE II.—COMPARISON OF ERRORS IN THE MEASUREMENTS OF A 50 P.P.M. CHROMIUM SAMPLE UNDER VARIOUS INSTRUMENTAL CONDITIONS

Conditions, 10 Determinations Each	Ratio Peak to Background	Net Peak Height, c.p.s.	Counting Error— % P.E.[a]	Counting Error— s_c[b]	Observed Error, s_o[c]	Calculated Extraneous Error, s_e[d]
1. In air, scintillation counter, no reverter	1.334	31.8	3.16	1.49	1.89	1.16
2. In air, scintillation counter, with reverter	2.082	30.9	2.06	0.94	1.79	1.52
3. In air, flow counter, no reverter	3.151	44.3	1.41	0.925	1.58	0.80
4. In helium, flow counter, no reverter	4.412	142.4	0.71	1.50	1.17[e]	0
5. In helium, flow counter, with reverter	5.084	138.0	0.70	1.43	2.40	1.93

[a] % P.E. = Per cent probable error in net peak height = $67.5/(R-1)\sqrt{[R(R+1)]/N_t}$ where R = ratio of peak height to background rates, N_t = total number of counts at peak top, including background. [b] s_c = Net peak height standard deviation, c.p.s. = % P.E. × net peak height, c.p.s./67.5. [c] s_o = Net peak height standard deviation, c.p.s. = $\sqrt{[\Sigma(X_i - \bar{X})^2]/N - 1}$. [d] $s_e = \sqrt{s_o^2 - s_c^2}$. [e] Lower than what is anticipated from pure counting errors, but not by a statistically significant amount.

Common Errors and Optimum Conditions for the Determination of Lighter Elements. A Statistical Study.—The following study was designed to show the relative contributions to precision of the use of helium atmosphere, the flow counter, and the reverter, when considered individually as applied to low concentrations of a light element. In general, these are three major considerations for sources of aid to precision in any prospective procedure. This study was also designed to show the relative sizes of the various types of error, and their contributions under the experimental conditions employed.

Errors in X-ray emission analysis are conveniently divided into two groups. One group consists entirely of counting errors, which stem from the randomness observed when X-ray emission quanta are measured. A second group comprises all sources of error other than counting errors, such as poor reproducibility in resetting the goniometer (2θ setting), sample positioning, etc.

Counting errors can be controlled. The per cent standard counting error, for instance, approximates the reciprocal of the square root of the total number of counts involved. Thus, at a sacrifice of time, a counting rate based on more counts decreases this error by any desired amount.

For a truly critical comparison of instrumental conditions for a given determination, it is necessary to separate these errors and consider the "extraneous error," or that calculated quantity which remains after all counting errors are reduced to zero.

The results of such a procedure are shown in Table II. All measurements were made on a compressed sample of steroidal material containing 50 p.p.m. chromium. Tungsten radiation and the fixed counting time of 100 sec. were used. Five instrumental conditions, derived from combinations of three major variables which strongly affect emission measurements, were statistically evaluated by making 10 determinations for each.

The five conditions of Table II are arranged in increasing order of peak-to-background ratios. These ratios have often been reported as being useful as a rough measure of method precision. The present study belies this usage.

Comparison of the values for the calculated extraneous errors indicates condition 4 to be the one of choice, and further indicates the use of the flow counter to be the single most effective variable in gaining precision under the experimental conditions.

The reverter, often used to good advantage to discriminate against undesired wavelengths when interfering elements are present, was found to be of no advantage in the present study except in connection with the scintillation counter. Even though an increase in peak-to-background ratio was significant, Table II shows large reverter contribution to the extraneous error.

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Comparative Cardio-Inhibitory Effects of Substituted Cyclohexanols

By HAROLD H. SMOOKLER and JOHN J. DEFEO

Cyclohexanol and its derivatives 2-(*o*-tolyl), 2-(*m*-tolyl), 2-(*p*-tolyl), 2-(*o*-chlorophenyl), 2-(*m*-chlorophenyl), and 2-(*p*-chlorophenyl) were tested for cardioplegic effects on intact anesthetized rats, isolated guinea pig hearts, and isolated rabbit atria. Most of the compounds produced inhibitory effects varying from a slight depression to complete arrest. Evaluation of potency was based on the ability to produce arrest and the duration of arrest. In both series of substitutions it appears that the *trans*-2-*o*-derivatives are most potent with the *cis*-2-*o*-derivatives least potent. The 2-*m*- and 2-(*p*-chlorophenyl) compounds appear to be toxic to the myocardium as the hearts do not return to their normal level of activity. Mechanism studies indicate an antagonistic effect between these compounds and calcium.

CARDIOPLEGIC AGENTS have become of interest in the past several years with the advances made in "open heart" surgery techniques employing extracorporeal circulation. Such agents would be of great help in securing a motionless field for surgical repair of various complicated cardiac defects.

Elective cardiac arrest has been attempted with such substances as potassium citrate and acetylcholine (1–4). Opinions on the use of cardioplegic agents vary from skepticism to acceptance for routine use.

Although cyclohexanol has never been reported as having cardioplegic properties, it is evident from various reports that certain derivatives bring about such an effect.

In 1957 (5) it was reported that 2-(*o*-methyl phenyl)-cyclohexanol [actually 2-(*o*-tolyl) cyclohexanol] had been able to arrest the heart of a dog but recovery was unsuccessful.

Huitric (6) and his co-workers published a note concerning the production of mechanical cardiac arrest with 2-(*o*-tolyl)-cyclohexanol. During the course of his work, Huitric had synthesized a series of substituted cyclohexanols (Fig. 1) which were used for this study of the comparative effects of the various substitutions on their ability to produce cardiac arrest.

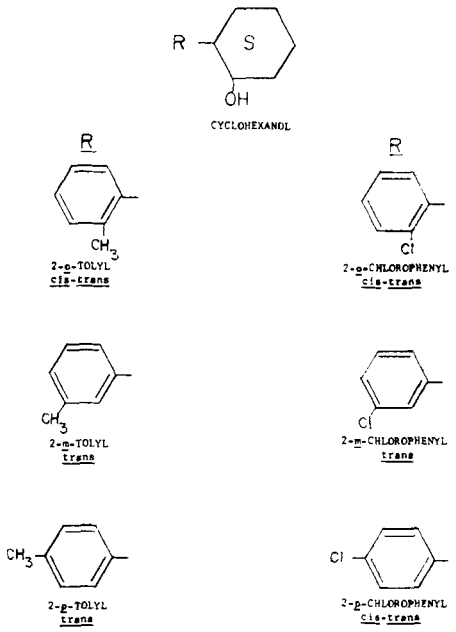


Fig. 1.—Cyclohexanol and its derivatives.

METHODS

The compounds whose structures appear in Fig. 1 have a very low solubility in water. In the tolyl series the *trans*-2-(*p*-tolyl) compound was the only solid, while others were liquid. In the chlorophenyl series, all were solid except the *cis*-2-(*o*-chlorophenyl) compound which was a liquid.

The solid materials were dissolved in 10–50% propylene glycol-water mixtures. An aliquot was measured and diluted to a standard concentration and volume. The liquid forms were mixed with water in order to obtain saturated solutions. A portion of the saturated solution was filtered and the filtrate was employed for testing.

The solubilities of the liquid substances were determined and are listed in Table I. Cyclohexanol was dissolved in water in varying concentrations.

Blood Pressure Studies.—The experiments were conducted in albino rats of both sexes. They were anesthetized with an intraperitoneal injection of 1.2 Gm./Kg. of urethan. The effects were recorded by direct cannulation of the carotid artery through a mercury manometer connected by plastic tubing filled with 7.5% sodium citrate solution. The compounds were administered intravenously. These studies were employed only to determine the rapidity

Received April 28, 1961, from the Department of Pharmacology, College of Pharmacy, University of Rhode Island, Kingston.
Accepted for publication October 20, 1961.
Supported in part by a grant from the Rhode Island Heart Association.
Abstracted from a thesis submitted by H. H. Smookler in partial fulfillment of the requirements for the M.S. degree, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

TABLE I.—WATER SOLUBILITY OF CYCLOHEXANOL AND ITS LIQUID 2-ARYL SUBSTITUTION PRODUCTS

Compound	Physical State	Water Solubility, mg./ml.
Cyclohexanol	liquid	5.67 at 15°[a]
trans-2-(*o*-Tolyl)cyclohexanol	liquid	0.8[b]
cis-2-(*o*-Tolyl)cyclohexanol	liquid	0.5
trans-2-(*m*-Tolyl)cyclohexanol	liquid	1.0
cis-2-(*o*-Chlorophenyl)cyclohexanol	liquid	0.2

[a] Handbook of Chemistry and Physics, 35th ed. (1953–1954). [b] Value reported by Huitric, *et al.* (6).

of effect in the intact animal. Those compounds dissolved in the propylene glycol could not be tested, as the solvent has a pronounced hypotensive effect and thus would mask the effect of the compounds.

Guinea Pig Heart Perfusion.—Guinea pigs of either sex were sacrified with a blow to the head. The heart was removed rapidly and flushed with the perfusing solution. It was then suspended in an Anderson-Craver perfusion apparatus by tying a glass cannula into the aorta. Chenoweth's solution (7) modified by replacing magnesium chloride with an equivalent amount of magnesium sulfate at 37.5°, and saturated with a steady flow of a 95% oxygen, 5% carbon dioxide mixture was employed as the perfusing fluid. The total volume of fluid containing the test compound injected into the heart was kept constant at 2 ml. This placed a limit on the upper level of dosage for the liquid compounds because of their poor solubility. An equivalent mixture of propylene glycol and water was used as a control prior to each injection of the compounds dissolved in this material in order to determine its effects.

The magnitude of effect, the duration of the effect, and the time for recovery to 50% of the normal amplitude was determined following each injection of the test compounds.

Isolated Rabbit Atria.—Rabbits were sacrificed and their hearts removed rapidly. The atria were resected intact and suspended in a (70 ml.) tissue bath containing the same nutrient solution as used in the perfusion experiments. The temperature was maintained at 32° in order to reduce the rate and to increase the amplitude of contractions (8).

A 4.0% solution of calcium chloride was used to determine possible interactions with *trans*-2-(*o*-tolyl)-cyclohexanol, which was selected because it appeared to be the most effective of the compounds tested. All drugs were allowed to act for exactly 3-minute periods before adding other agents or washing.

trans-2-(*o*-Tolyl)-cyclohexanol produced an effect lasting indefinitely when not washed. Calcium chloride produced an effect that lasted for at least 30 minutes if not washed.

The experiments were conducted so that the effects of the experimental compounds were recorded well within the above periods in order to assure ourselves that the effects were due to the drugs alone.

RESULTS

Cyclohexanol in doses of 5 mg./Kg. to 100 mg./Kg. produced a drop in mean blood pressure ranging from 20 to 90% of the normal.

trans-2-(*o*-Tolyl)-cyclohexanol in doses of 0.5 to 4.0 mg./Kg. produced a drop in mean blood pressure of 38 to 83%.

cis-2-(*o*-Tolyl)-cyclohexanol in doses of 1.0 to 3.0 mg./Kg. produced a drop in mean blood pressure of 27 to 74%.

trans-2-(*m*-Tolyl)-cyclohexanol in doses of 1.0 to 6.0 mg./Kg. produced a drop in mean blood pressure of 18 to 99%.

cis-2-(*o*-Chlorophenyl) in doses of 0.5 to 1.5 mg./Kg. produced a drop in mean blood pressure of 37 to 91%.

In all of the above experiments death occurred at the highest dose when this quantity was readministered in the same animal. At the conclusion of all of the tests, urine was aspirated from the bladder and hematuria was found in every case.

The results obtained in the guinea pig heart perfusion studies are shown in Table II. It should be noted that a normal heart will contract from 2 to 3 hours in the apparatus before signs of failure appear. In all of the experiments the compounds acted within 1 minute.

Recovery was allowed to occur spontaneously in order to compare the rates of recovery from the effects of the various compounds. Recovery to a 50% level of the original amplitude was variable, ranging from 3 to 29 seconds for cyclohexanol to 34 to 430 seconds for *trans*-2-(*o*-tolyl)-cyclohexanol.

TABLE II.—COMPARATIVE EFFECTS OF CYCLOHEXANOL AND ITS DERIVATIVES ON THE ISOLATED GUINEA PIG HEART

Compound	Minimum[a] Effective Dose, mg.	Cardiac Arrest Arrests/ Trials	Duration of Arrest Mean and Range, sec.	Mean % Depression ± S.D. (if no arrest)
Cyclohexanol	8.0	7/7	18(9–58)	...
cis-2-*o*-Tolylcyclohexanol	0.5[b]	0/25	...	63 ± 17
trans-2-*o*-Tolylcyclohexanol	0.4	5/6	12(7–14)	85
trans-2-*m*-Tolylcyclohexanol	1.0	5/18	33(11–53)	85 ± 7
trans-2-*p*-Tolylcyclohexanol	1.5	7/16	170(37–275)	88 ± 6
cis-2-*o*-Chlorophenylcyclohexanol	0.2[b]	0/12	...	26 ± 12–93(4)[c]
trans-2-*o*-Chlorophenylcyclohexanol	1.0	9/11	88(29–167)	93 ± 1
trans-2-*m*-Chlorophenylcyclohexanol	1.0	3/8	197(62–284)	62 ± 16
cis-2-*p*-Chlorophenylcyclohexanol	2.0	3/6	262(148–324)	71 ± 18–100(1)[d]
trans-2-*p*-Chlorophenylcyclohexanol	2.0[e]	4/4	442(182–645)	...

[a] Minimum dose which will produce cardiac arrest. [b] Maximum allowable dose injected but not the minimum effective dose. [c] Stimulation occurred in 4 of 12 doses with a mean of 93%. [d] Stimulation occurred in 1 of 6 doses. [e] Toxic dose.

There is an indication of toxicity produced by the *trans*-2-(chlorophenyl) compounds since many of the hearts did not return to normal following the arrest periods.

cis-2-(*o*-Chlorophenyl)-cyclohexanol acted in a manner differing from the other compounds in that it produced a stimulating effect up to 93% of the normal in some hearts. In other cases it was only slightly depressant.

trans-2-(*o*-Tolyl)-cyclohexanol was selected for preliminary mechanism studies due to its reproducible action. A dose of 0.6 mg. was found to be immediately effective in reducing the amplitude of the atrial contractions by 50% without apparently affecting the rate.

It was noted that 4 mg. of calcium chloride in the 70-ml. bath was sufficient to increase the amplitude of the atrial contractions by 100%. When *trans*-2-(*o*-tolyl)-cyclohexanol (0.6 mg.) had reduced the amplitude of contractions by 50% it was found that it required three successive doses of 8 mg. each of calcium chloride over 9 minutes in order to restore the amplitude of contraction, stepwise, to a normal level. When a total of the above amounts (24 mg.) was administered at one time there was a continuous recovery within 3 minutes.

In other tests, when *trans*-2-(*o*-tolyl)-cyclohexanol (0.6 mg.) followed calcium chloride (4 mg.) it was observed that the onset of action was delayed several minutes and the inhibitory effect which followed only overcame the effect of the calcium, thus restoring the amplitude of contractions to a normal or slightly below normal level.

DISCUSSION

The *trans* isomers of these compounds are those in which both the substituted groups and the hydroxyl group occupy equatorial positions on the cyclohexane ring. The *cis* isomers are those where the substituted groups are equatorial and the hydroxyl group is axial to the cyclohexane ring (9).

It is evident that cyclohexanol and its liquid 2-aryl derivatives can produce an immediate drop in blood pressure. The magnitude of the effect is somewhat proportional to the dose. The substitution of a tolyl or chlorophenyl moiety in the number 2 position of the cyclohexanol ring increases the hypotensive activity from 6 to 40 times. This is no doubt due to the inhibitory effect of these substitutions on the myocardium as was shown in the isolated heart tests.

At the dose levels employed in these tests it appears that substitutions at the 2 position on the cyclohexanol structure increase the cardio-inhibitory effect from 4 to 20 times (see Table II). The *trans*-2-(*o*-tolyl) substitution is apparently the most effective in producing arrest at the lowest dose followed by a good recovery. The *cis* isomers of both the (*o*-tolyl) and (*o*-chlorophenyl) compounds are the least depressant, while the *trans* isomers of these compounds are the most effective in their respective series. In both series it appears that the inhibitory effect is enhanced by shifting the methyl on the tolyl and the chlorine on the chlorophenyl from *para* to *meta* to *ortho* positions, respectively.

Tentative conclusions indicate that the tolyl derivatives are less toxic than the chlorophenyl derivatives as evidenced by the failure of most hearts to recover from the effects of the latter compounds.

These facts indicate the importance of the spatial configuration of these compounds in producing their cardio-inhibitory actions.

The antagonism between the calcium ion and *trans*-2-(*o*-tolyl)-cyclohexanol is apparently of a physiological type. The 2-(*o*-tolyl)-cyclohexanol action appears to be the most potent as the same amount of this compound was able to counteract the effect of various amounts of calcium. However, when enough calcium was added to the bath following the depressant effect, it was able to restore the contractions of the atria to a normal level. The major effect of calcium was to increase the latent period of 2-(*o*-tolyl)-cyclohexanol.

SUMMARY

1. Tolyl and chlorophenyl substitutions at the 2 position of the cyclohexanol structure enhance the cardio-inhibitory effect of this compound.

2. The *cis* isomers of the (*o*-tolyl) and (*o*-chlorophenyl) compounds are least effective, whereas the *trans* isomers of these compounds are the most effective in producing cardio-inhibitory effects. The inhibitory effect increases with the position of the methyl and chlorine from *para* to *meta* to *ortho*.

3. The chlorophenyl substituted derivatives appear to exhibit a greater cardio-toxicity than the tolyl derivatives.

4. Calcium delays the onset of action of 2-(*o*-tolyl)-cyclohexanol and antagonizes its effect when administered after the depressant action of the compound has occurred.

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Effects of β-Diethylaminoethyl Diphenylpropylacetate Hydrochloride on Three Convulsants and on Propallylonal

By HARALD G. O. HOLCK, GALE E. DEMAREE†, and HIDEKO KATAYAMA KAJI‡

β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A) increased the LD_{50}s of nikethamide, pentylenetetrazol, and picrotoxin in albino mice when given intraperitoneally 1 hour earlier. The maximum action of picrotoxin in mice subcutaneously pretreated with SKF 525-A developed in about 4 hours; action was still marked at 8, but largely absent at 16 hours. SKF 525-A induced full effect when given intraperitoneally immediately before picrotoxin. It also markedly increased the potency of picrotoxin in normal and in barbitalized rats, using a multiple injection technique. However, SKF 525-A did not influence the toxicity of pentylenetetrazol in normal or barbitalized rats employing this technique. Barbital greatly increased the LD_{50}s of pentylenetetrazol and picrotoxin in rats, as shown by the multiple injection method. SKF 525-A markedly prolonged the hypnotic effect of propallylonal without increasing the number of delayed deaths. Its use is recommended for biotransformation studies of the four substances used.

BETA-diethylaminoethyl diphenylpropylacetate hydrochloride, hereafter referred to as compound SKF 525-A, has been shown to potentiate or prolong the action of a great variety of drugs. Cook, *et al.* (1, 2), in mice and rats, demonstrated that it prolonged the action of secobarbital, pentobarbital, hexobarbital, amobarbital, butethal, hexothal, phenobarbital, and chloral hydrate; however, barbital and thiopental depression in mice was not altered. On the other hand, Achor and Geiling (3), administering SKF 525-A intraperitoneally rather than orally, obtained prolongation of thiopental hypnotic action using similar and higher dosage of SKF 525-A. Macko, *et al.* (4), reported that this compound increased the central nervous system stimulation of *d*-amphetamine and strychnine and greatly increased the toxicity of the latter. Loewe (5) noted that the CNS-stimulating properties of yohimbine upon the butallylonal-yohimbine ejaculatory effect in mice was augmented by SKF 525-A. Axelrod and co-workers (6) and Cooper, *et al.* (7), reported that much of the effect of SKF 525-A is due to its ability to inhibit the biotransformation of the drugs in question. It becomes widely localized in rat organ tissues, especially fat, is present in the body after 24 hours, and is practically all metabolized *in vivo* (6). Swinyard and collaborators (8) have shown potentiation of the action of a series of antiepileptic drugs by SKF 525-A; however, the potentiation was not considered due entirely to inhibition of biotransformation of these drugs inasmuch as SKF 525-A possesses some anticonvulsant activity of its own in high dosage.

SKF 525-A is effective *in vivo* when administered up to 20 hours prior to the drugs whose effects are capable of being influenced; also, prolongation of hexobarbital depression could be demonstrated by administering SKF 525-A during hexobarbital hypnosis, but not after recovery (1, 2). It is equally potent when administered orally, intraperitoneally or intravenously (2). A suitable intraperitoneal and oral dose for mice and rats is given as 50 mg./Kg., and the optimum pretreatment time as 40 to 60 minutes prior to the administration of the drug under study (1).

The present series of experiments was designed to obtain information concerning the effects of SKF 525-A upon the median lethal doses of nikethamide, pentylenetetrazol, and picrotoxin in mice. Special attention was given to methods of administration, time interval for pretreatment, and duration of action with picrotoxin. Tests were also conducted in rats to note what influence barbital anesthesia might have upon the effect of SKF 525-A upon pentylenetetrazol and picrotoxin toxicity. Finally, the effect of SKF 525-A upon the duration of depression, incidence of delayed death, and sex variation with propallylonal in rats was studied.

Received October 9, 1961, from the Department of Pharmacology, College of Pharmacy, University of Nebraska, Lincoln.

Accepted for publication December 15, 1961.

Presented in part to the Scientific Section, A.PH.A., Miami Beach meeting, May 1955.

The major portions of this paper are taken from the Master's thesis of Gale E. Demaree.

We are indebted to the Smith Kline & French Research Laboratories for donating SKF 525-A; to Eli Lilly and Co. for picrotoxin; to Bilhuber-Knoll Corp. for Metrazol (pentylenetetrazol); to Ciba Pharmaceutical Products for Cardiazol (nikethamide); and to Miles-Ames Research Division for Nostal (propallylonal). We also wish to thank Messrs. David Sjogren and Loren Braun for aid in conducting some of the experiments and to Deans J. B. Burt and R. D. Gibson for constructive criticism of the manuscript.

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EXPERIMENTAL

General.—Male mice of Swiss albino stock, weighing 16–26 Gm. and randomized according to weight within each suitably sized group, were used in the niketharnide and picrotoxin experiments. With pentylenetetrazol, six males and six females were used in each group. All mice were kept for at least 1 week in temperature-controlled animal quarters on a diet of Purina Laboratory Chow and water *ad libitum* until the time of experimentation. After injection the mice were kept in individual large glass beakers and the room temperature maintained at 25 ± 0.5°. SKF 525-A was administered as a 1.0% solution in distilled water. A 3% solution of niketharnide, a 1% solution of pentylenetetrazol, or a 0.05% solution of picrotoxin was employed; distilled water served as the diluent. Suitable logarithmic dosages were utilized and the median convulsive and lethal doses (CD_{50}s and LD_{50}s) were determined by the Reed-Muench and Pizzi methods, using logarithms (10, 11). An equivalent volume of 0.9% sodium chloride solution was injected into the control groups. Whenever an interval of 3 hours or longer occurred between the injection of SKF 525-A and the picrotoxin, the mice were returned to the stock cages immediately after the administration of SKF 525-A and allowed food and water until 1 hour prior to the scheduled administration of the pictrotoxin. They were then reweighed, placed in individual containers, and the picrotoxin dose calculated from the final weights. In a few cases, when a very large number of mice were under study, it was impractical to complete a series in one day so the series was continued on a later day. All intraperitoneal injections were made in the upper left quadrant of the abdomen, while all subcutaneous injections were made under the loose skin of the lower back.

In the further studies of the effects of SKF 525-A upon pentylenetetrazol and picrotoxin, Holtzman male rats in groups of 10 were employed to determine the CD_{50}s and LD_{50}s in normal and barbitalized animals. Each analeptic was injected subcutaneously every 10 minutes, using ten different sites in rotation according to the technique previously developed by Holck (12). In case of pentylenetetrazol, 200 mg./Kg. of barbital sodium (10% solution) or 2 ml./Kg. of saline were injected intramuscularly 10 minutes prior to injecting 50 mg./Kg. (5% solution) of SKF 525-A or 1 ml./Kg. of saline intraperitoneally. Sixty minutes later an initial dose of 9 mg./Kg. (0.9% solution) of pentylenetetrazol was administered to the nonbarbitalized rats and 25 mg./Kg. (5% solution) to the barbitalized rats and their controls. Barbital was chosen as the hypnotic agent because its action is not prolonged by pretreatment with SKF 525-A (2); it also has more recently been shown that SKF 525-A does not alter its induction time (13). The age of the first two groups of rats was about 5 months, and of the last three groups about 10 months.

In the picrotoxin tests, the barbital sodium dose was 225 mg./Kg. (11.25% solution); the dose of picrotoxin per injection was 0.7 mg./Kg. (0.07% solution) for nonbarbitalized rats and 1 mg./Kg. (0.1% solution) for barbitalized rats. It was necessary to increase the dose of picrotoxin because tolerance developed to the lower dose. The ages of the animals in the two sets of tests were 3 and nearly 5 months, respectively. All rats were kept under conditions similar to those previously described for the mice.

In the studies with propallylonal, this drug was converted to its sodium salt by the addition of an appropriate amount of 0.2 *N* sodium hydroxide solution and diluted to the desired volume with freshly boiled, triple distilled water. Holtzman rats of both sexes were used, because female rats in previous studies usually have proven more sensitive to propallylonal than the males. Rats, 4–6 months old, were used in the first experiment in which 30 mg./Kg. (3% solution) of propallylonal were injected subcutaneously as a single dose. In the experiment using a 50 mg./Kg. dose, a 4% solution was employed and injected intraperitoneally; these rats were 4 months old. In both tests the rats were fasted overnight but allowed water. Intraperitoneal administration of 50 mg./Kg. (2.5% solution in the first, 5% in the second test) of SKF 525-A, or of an equal volume of saline, was made 1 hour prior to the barbiturate medication. The animals were observed during 6 days following propallylonal administration for incidence of delayed death.

RESULTS

Concomitant with administering the analeptics to mice it was ascertained that the stock used had a resistance to SKF 525-A quite similar to that for intraperitoneal administration reported by Cook, *et al.* (1). Thus, with a 1% solution, a temperature of 25° and 10 male mice per group, none died with 100, 5 with 141, and all with 200 mg./Kg. However, with subcutaneous injection the resistance was much higher in that 10 mice survived 100, 200, and 400 mg./Kg., respectively; a 2% solution was used. The results of the experiments with the analeptics in mice and rats are presented in Tables I and II.

Following subcutaneous administration of 30 mg./Kg. of propallylonal, only $^3/_{24}$ rats pretreated with SKF 525-A lost the righting reflex compared with none of the controls. No delayed deaths occurred in either group. With 50 mg./Kg. of propallylonal given intraperitoneally, the righting reflex was recovered in 5 control females in 186 ± 36 and in 5 pretreated ones in 345 ± 15 minutes; $P < 0.01$. In the males the minutes were 73 ± 6.3 for 4 out of 5 controls and 238 ± 24 for 5 pretreated animals; $P < 0.01$. P was about 0.03 for the sex variation in the controls and 0.02 in the pretreated rats. Although the depression time was significantly prolonged by SKF 525-A, the number of delayed deaths was not increased, being $^2/_5$ in the control and pretreated females, respectively, and $^1/_5$ in similar male groups.

DISCUSSION

An outstanding finding of our studies upon albino mice was that the ratio of the LD_{50} of the controls over that of those pretreated with SKF 525-A was smaller for pentylenetetrazol and for niketharnide than for picrotoxin. It was 1.24 for pentylenetetrazol and 1.32 for niketharnide, as compared with 2.63 and 2.81, respectively, in two tests with picrotoxin. The last ratios are in good agreement with the 2.6 reported by Macko, *et al.* (4), for strychnine.

TABLE I.—EFFECT OF β-DIETHYLAMINOETHYL DIPHENYLPROPYLACETATE HYDROCHLORIDE (SKF 525-A) ON THE INCIDENCE OF ACUTE DEATH WITH PENTYLENETETRAZOL, NIKETHAMIDE, AND PICROTOXIN IN ADULT ALBINO MICE

	SKF 525-A, mg./Kg.	Route	Hours after SKF 525-A	No. of Mice/ Mice per Group[a]	LD_{50} ± S.E., mg./Kg.	*P*	
				Pentylenetetrazol			
A	Control	i.p.	1	60/12	77.5 ± 3.4	...	
B	50	i.p.	1	72/12	62.1 ± 2.7	<0.005	
				Nikethamide			
A	Control	i.p.	1	40/10	325 ± 19	...	
B	25	i.p.	1	40/10	283 ± 17	>0.1	A *vs.* B
C	Control	i.p.	1	50/10	327 ± 22	...	
D	50	i.p.	1	40/10	248 ± 15	<0.02	C *vs.* D
				Picrotoxin			
A	Control	i.p.	1	52/13	6.24 ± 0.25	...	
B	25	i.p.	1	50/10	2.54 ± 0.19	<0.001	A *vs.* B
C	Control	i.p.	1	60/10	6.54 ± 0.38	...	
D	50	i.p.	1	60/10	2.47 ± 0.16	<0.001	C *vs.* D
E	Control	i.p.	1	50/10	2.70 ± 0.21[b]	...	
F	50	i.p.	1	70/10	0.84 ± 0.07[b]	<0.001	E *vs.* F
A	50	s.c.	1	40/10	4.42 ± 0.32	...	
B	50	s.c.	2	40/10	3.53 ± 0.27	<0.07	A *vs.* B
C	50	s.c.	4	50/10	3.17 ± 0.20	<0.01	A *vs.* C
D	50	s.c.	8	50/10	4.05 ± 0.23	<0.02	C *vs.* D
E	50	s.c.	16	30/10	5.82 ± 0.30	<0.001	D *vs.* E
A	Control	i.p.	1	36/12	7.13 ± 0.38	...	
B	50	i.p.	3	48/12	3.31 ± 0.17	<0.001	A *vs.* B
C	50	s.c.	3	60/12	3.54 ± 0.17	<0.001	A *vs.* C
D	50	s.c.	6	60/12	3.75 ± 0.21	<0.001	A *vs.* D
A	Control	i.p.	0	45/15	5.96 ± 0.26	...	
B	50	i.p.	0	60/15	2.23 ± 0.10	<0.001	A *vs.* B
C	50	i.p.	1	60/15	2.10 ± 0.10	<0.001	A *vs.* C

[a] Numbers of mice in groups not showing either 100% negative or 100% positive responses/number of mice per dosage group. Represent CD_{50}s.

TABLE II.—EFFECT OF β-DIETHYLAMINOETHYL DIPHENYLPROPYLACETATE HYDROCHLORIDE (SKF 525-A) ON THE INCIDENCE OF CONVULSIONS, DEVELOPMENT OF TOLERANCE, AND LETHAL DOSE WITH PENTYLENETETRAZOL AND PICROTOXIN, USING THE MULTIPLE INJECTION METHOD IN NORMAL AND BARBITALIZED ADULT MALE ALBINO RATS

Injection, mg./Kg.	Barbital Sodium, mg./Kg.	SKF 525-A, mg./Kg.	CD_{50} ± S.E., mg./Kg.	LD_{50} ± S.E., mg./Kg.	*P*	
			Pentylenetetrazol			
9	None	None	...	134 ± 5.9[a]	...	
9	None	50	...	119 ± 8.5[a]	>0.1	
25	None	None	75 ± 6.5	130 ± 19.6[a]	...	
25–50[b]	200	None	...	1085	...	
25–50[b]	200	50	...	1105	...	
			Picrotoxin			
0.7	None	None	4.97 ± 0.49	9.17 ± 0.32	<0.001	(CD_{50}s)
0.7	None	50	3.15 ± 0.12	5.46 ± 0.17	<0.001	(LD_{50}s)
1.0	225	None	[c]	[c]	...	
1.0	225	50	7.8 ± 0.13	12.4 ± 0.22	...	

[a] There was evidence of development of tolerance in a few of the rats. [b] No convulsions occurred in either group after 12 injections (300 mg./Kg.), hence the dose per injection was raised to 50 mg./Kg. [c] Only one rat convulsed and none died after 20 injections (20 mg./Kg.). All 10 rats made uneventful recoveries and were alive 5 days later. The average doses producing recovery of the righting reflex were 6.0 ± 0.45 mg./Kg. for the controls and 6.5 ± 0.27 mg./Kg. for the SKF 525-A premedicated rats; $P < 0.3$.

The results with picrotoxin agree with those of Cook, *et al.* (1), in that the action occurred almost immediately after intraperitoneal administration of SKF 525-A and was fully developed in 1 hour; it lasted more than 16 hours after subcutaneous administration. Although marked action was noted after 1 hour, maximum action with subcutaneous pretreatment with SKF 525-A did not occur until several hours later, indicating a slower absorption of SKF 525-A after this method of injection. The much more gradual absorption is also indicated by the mentioned much higher fatal dose of SKF 525-A with subcutaneous compared with intraperitoneal administration. The results with albino rats also agree in principle with those of Cook, *et al.* (2), in that the increase in duration of hypnotic action after propallylonal occurred without increasing the number of delayed deaths; however, the prolongation of depression was much less marked than in case of hexobarbital. Tolerance to pentylenetet-

razol occurred with and without SKF 525-A pretreatment in all of the barbitalized rats and in some of the normals, using a multiple injection technique. Cook, *et al.* (9), have reported a similar absence of interference with development of tolerance to morphine by SKF 525-A; it did not change its LD_{50}.

On the other hand, pretreatment with SKF 525-A markedly lowered the fatal dose of picrotoxin in barbitalized rats and significantly decreased the convulsive and fatal doses in normal rats. The results with barbitalized rats, pretreated with SKF 525-A, were unusually uniform in that the standard errors were less than 1.8% of the CD_{50}s and LD_{50}s of picrotoxin, compared with 3.5% and 3.1% in normal, pretreated rats.

The major factor of SKF 525-A effect is due to the inhibition of biotransformation of substances susceptible to its influence. The extensive review by Brodie (14) involving, in part, numerous *in vivo* and *in vitro* studies by his own staff, indicates that a similar inhibitory effect by SKF 525-A may be exerted on a variety of metabolic pathways or, as stated by Cooper, *et al.* (7), it inhibits a factor or factors possessed in common by a diversity of drug enzyme systems. Depression of bioacetylation and of acetylcholinesterase activity has since been reported by Kuriaki and Marumo (15). The mechanism involved in the case of the three analeptic drugs tested by us remains to be determined. With propallylonal, SKF 525-A may prevent the replacement of Br by an OH group and subsequent molecular rearrangement of the ketone form to give an acetonyl group, which leads to formation of a barbiturate that is much weaker and slower in action than the β-bromallyl derivative (16, 17).

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Temperature-Induced Rheological Variability in an Emulsion System

By JAMES C. BOYLAN, H. GEORGE DeKAY, and GILBERT S. BANKER

A study has been made on the effect of emulsification temperature and cooling rate on certain physical properties of a beeswax-mineral oil emulsion. The emulsion, which was prepared at five temperatures and cooled at three rates, was evaluated for plastic viscosity, thixotropy, yield value, water separation rate, and initial average particle size. It was shown that both the emulsification temperature and cooling rate significantly altered these properties.

IN THE PAST few decades, emulsions have been studied from many viewpoints. Despite this, the formulation, preparation, and stability of most emulsion products remains, to a large extent, an empirical art. Investigations to date have failed to pinpoint the rheological characteristics of many emulsions.

It is generally agreed that an increase in temperature facilitates emulsification (1–3). One author (4) contends in rather vague and general terms that an emulsification temperature should be chosen which will give the optimum dispersion to the materials being emulsified. Spalton (5) specifies that a freshly prepared wax emulsion should be allowed to cool slowly since rapid cooling may result in a granular product. Wellman (6) found that the solubility of the soap, as controlled by temperature, is an important factor in emulsion formation. Several studies have been conducted attempting to relate viscosity of emulsions to temperature (7–9).

In the area of particle size and its effect on viscosity, Sherman (10) states that there is a frequent increase in viscosity when an emulsion is homogenized. He feels that this increase in viscosity is due to a decrease in particle size,

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Accepted for publication July 20, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961

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which leads to increased interfacial area and interaction among the globules.

The objective of this study was to determine the following effects of various emulsification temperatures and rates of cooling on a wax-containing emulsion: (*a*) the rheological properties (i.e., viscosity, yield value, and thixotropy), (*b*) the rate of creaming and/or separation of the phases, and (*c*) the particle size of the dispersed phase.

EXPERIMENTAL

Emulsion Formulation and Preparation.—A beeswax emulsion of the following composition was used throughout this study

	Amount, Gm.
White wax	80.0
Liquid petrolatum (heavy)	250.0
Sodium borate	11.0
Purified water	659.0
to make	1000.0

The emulsion formula was chosen on the basis of its simplicity and the wide cosmetic and pharmaceutical use of its ingredients.

The wax and liquid petrolatum were placed in a 2000-ml. stainless steel beaker (inside height 16.5 cm., inside diameter 12.5 cm.). The sodium borate and the water were placed in a 1000-ml. glass beaker. The aqueous phase was brought to the desired temperature by means of a bunsen burner. The temperature of the oil phase was controlled by means of a heating mantle,[1] regulated by a variac. When the desired temperature of both phases was reached, the aqueous phase was slowly added to the oil phase (which was still encased in the heating mantle). A model V-7 "lightin" mixer[2] was immediately started at 1875 r.p.m. and allowed to run for 15 seconds to achieve initial emulsification. The emulsion was then removed from the heating mantle and placed in a specially constructed air bath (see Fig. 1) which had been preset to cool the emulsion at one of the prescribed rates. A second "lightin" mixer within the air bath was started and run at 1800 ± 100 r.p.m. until the emulsion cooled. All emulsions were cooled in the air bath to 30°. With practice, the procedure and time required for enclosing the emulsion in the air bath became nearly constant.

Both mixers utilized a three-blade marine-type propeller, the length of each blade being 1.6 cm. The propeller was mounted on a 26.9-cm. shaft. In each case, the propeller was placed off center in the same quadrant and one-third of the distance from the bottom of the beaker relative to the height of the liquid, with the mixer shaft at an angle of about 25° from the perpendicular. A Stobatac[3] was used to standardize the mixer speeds. A small oscillating fan inside the air bath maintained air circulation and a 12-inch evaporating dish filled with distilled water minimized fluctuations in relative humidity during the experiment.

[1] Glas-col Apparatus Co., Terre Haute, Ind.
[2] Mixing Equipment Co., Inc., Rochester, N. Y.
[3] General Radio Corp., Chicago, Ill.

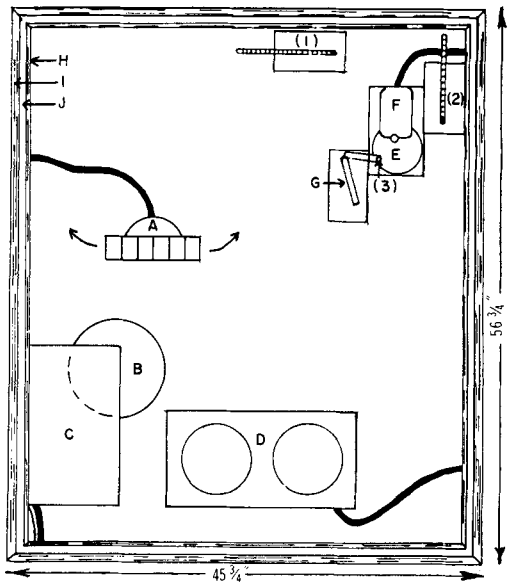


Fig. 1.—Top view of the air bath. The materials are: 3/4 and 3/8 inch plywood, glass wool (insulator), and double glass window above the mixer area. The inside of the roof and the sides are lined with aluminum foil. Thermometers, supported on ring-stands, are at points 1, 2, and 3. A, rotating fan; B, evaporating dish; C, refrigeration unit; D, hot plate; E, emulsion; F, mixer; G, magnifying mirror; H, 3/8 inch plywood; I, 3/4 inch plywood; J, 3/4 inch glass wool.

The emulsions were prepared at five temperatures and cooled at three rates (see Table I). When a sample was to be cooled slowly, the air bath was preset at 25 ± 0.5°. Since the heat lost by the emulsion upon cooling remained in the hermetically sealed air bath, the temperature inside the bath rose to 29–30°. If the emulsion was to be cooled at a moderate rate, the air bath was preset at 11 ± 1°. The temperature within the bath then rose to 14–16°. In the case of rapidly cooled products, the stainless steel beaker containing the emulsion was placed in a 10-inch water bath containing crushed ice. This was then introduced into the precooled air bath (11 ± 1°).

TABLE I.—SUMMARY OF EMULSION MANUFACTURE

Emulsion Notation[a]	Emulsification Temperatures, °C. Aqueous Phase	Wax Phase	Rate of Cooling to 30°, min.	Temperature when Placed in Air Bath, °C.
1-S	70	70	127	68
1-M	70	70	66	68
1-F	70	70	21.5	67
2-S	80	80	127.5	78
2-M	80	80	75	78
2-F	80	80	13.5	72
3-S	90	90	134	85
3-M	90	90	78	85
3-F	90	90	15.5	80
4-S	70	90	127	74
4-M	70	90	72.5	73
4-F	70	90	16	70
5-S	90	70	128	80
5-M	90	70	76	80
5-F	90	70	15.3	75

[a] S, slow cooled; M, medium cooled; F, fast cooled.

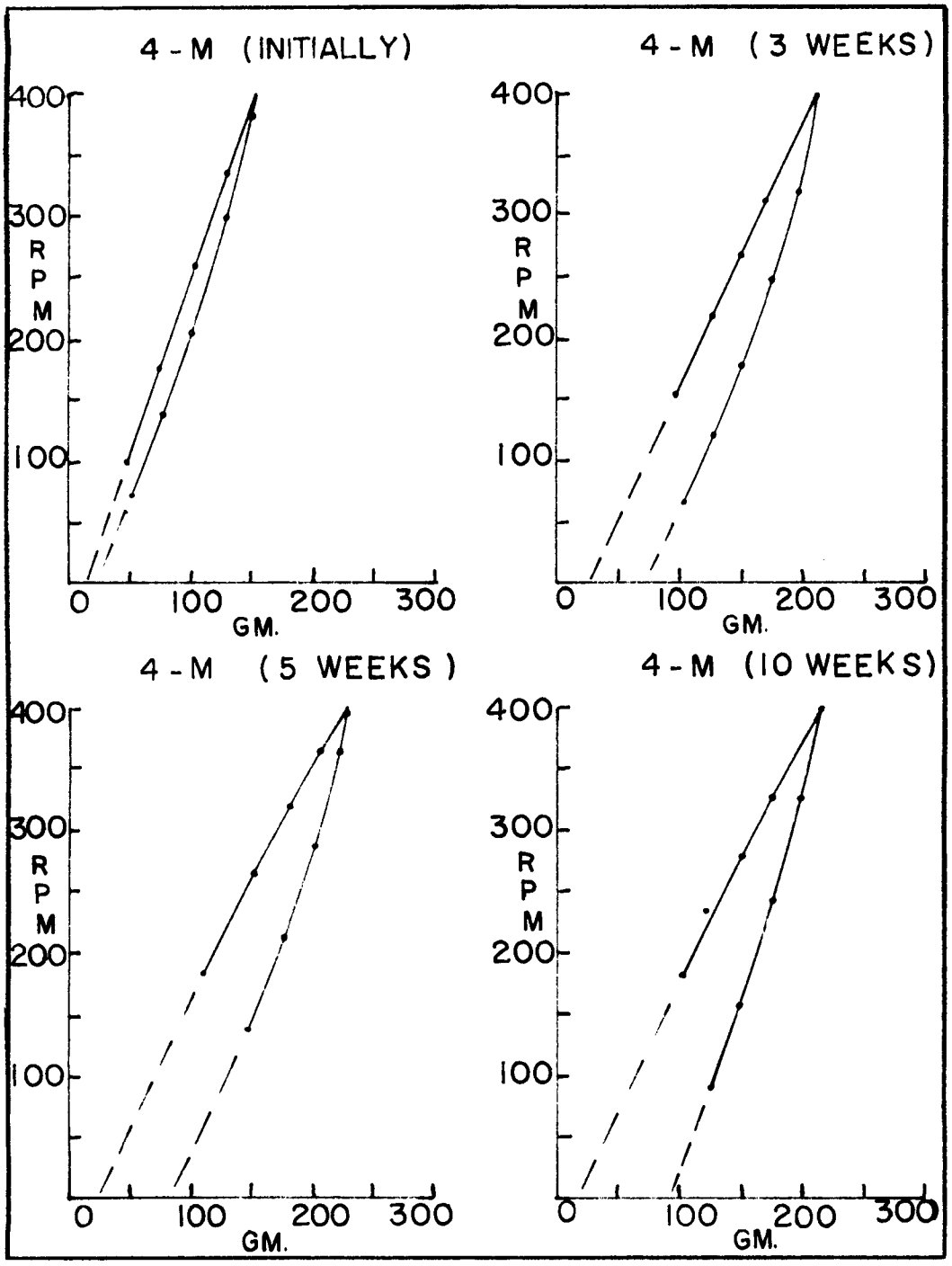


Fig. 2.—Emulsion 4-M rheograms showing the effect of aging.

Rheological Evaluation.—The viscometer chosen for this study was a Stormer viscometer provided with the modified cup and bob as suggested by Fischer (11). A weight hanger with various slotted weights provided the shearing stress. A constant temperature water bath maintained the sample temperature at 25 ± 0.1°. A calibration curve for the cup and bob was prepared using seven N.B.S. standard viscosity oils. The emulsion sample was placed in the cup and deaerated by gentle suction. The cup was carefully raised into position, taking care not to entrap air under the bob. The system was then conditioned for 2 hours before beginning a run. The rheogram was obtained by adding successively increasing weights to the hanger. A point on the curve was obtained by noting the

TABLE II.—EFFECT OF EMULSIFICATION TEMPERATURE, RATE OF COOLING, AND SAMPLE AGE ON THE RHEOLOGY OF A WAX-CONTAINING EMULSION

Emulsion Notation[a]	Rheological Parameter	0 Days[b]	1 wk.	3 wk.	5 wk.	6 wk.	10 wk.
1-S	V[c]	59.25	62.87	82.69	78.00	85.72	92.10
	T[d]	2.86	1.86	6.63	5.75	9.14	9.02
	f[e]	22.74	25.63	39.00	29.62	35.34	37.26
1-M	V	68.52	66.96	82.69	96.76	98.17	122.1
	T	3.67	6.20	8.95	14.85	14.71	20.15
	f	32.18	27.57	34.35	43.87	41.10	25.43
1-F[g]	V	70.93	67.95	72.58	92.73	93.80	95.01
	T	7.36	8.00	8.21	17.04	17.50	22.00
	f	34.08	33.10	32.29	57.78	58.16	59.50
2-S	V	46.48	41.77	69.55	76.58	80.01	71.96
	T	0.89	0.95	4.86	8.16	8.09	8.22
	f	17.86	15.38	34.98	42.53	37.06	36.88
2-M	V	61.75	77.58	71.54	79.63	89.64	85.72
	T	3.72	7.00	5.98	7.98	10.68	11.47
	f	25.02	22.20	23.96	27.80	21.39	30.69
3-S	V	40.10	45.45	81.64	80.63	80.63	88.09
	T	0.77	1.31	7.05	5.75	5.48	10.14
	f	13.20	12.48	32.46	38.01	38.01	30.69
3-M	V	64.30	78.00	89.64	94.19	93.80	96.76
	T	3.68	6.97	14.09	15.54	14.50	18.03
	f	25.68	27.77	39.99	37.32	38.25	27.07
4-S	V	41.01	76.58	77.00	74.96	70.93	84.67
	T	1.00	6.04	6.41	6.41	5.92	6.84
	f	15.02	40.68	30.53	34.17	35.00	46.45
4-M	V	62.83	92.10	90.07	85.10	90.65	87.61
	T	3.68	12.84	11.77	15.37	14.70	15.48
	f	28.37	18.63	42.80	52.98	43.74	35.34
5-S	V	41.01	65.44	83.08	86.11	81.64	89.06
	T	1.33	4.76	8.18	8.27	9.00	9.30
	f	17.67	34.85	46.43	41.85	40.81	39.06
5-M	V	66.96	91.62	103.8	105.9	95.16	101.8
	T	3.92	13.19	18.51	18.26	16.35	15.07
	f	25.73	40.94	29.95	60.91	42.92	47.69

[a] See Table I for the relationship between the emulsion notation and the method of manufacture. [b] The first rheograms were prepared immediately after manufacture and cooling of the emulsion samples. [c] Plastic viscosity in centipoises. [d] Thixotropic area in square inches. [e] Yield value in dynes/cm.^{2}. [g] Rheological data for emulsion samples 2-F to 5-F could not be obtained due to the wax aggregates present.

time required for a given weight to drive the bob through 100 revolutions. This value was then converted to r.p.m. When a maximum of 380 to 410 r.p.m. was reached, the weight on the hanger was reduced in increments. An interval of 60 seconds was observed between each point on the curve. The calculations necessary to obtain the instrumental constants, plastic viscosity, and yield value were identical to those of Fischer (11). The thixotropic area was measured directly in square inches with a compensating planimeter.[4] At least two samples of each emulsion were run immediately after manufacture, and after 1, 3, 5, 6, and 10 weeks, the samples being stored at room temperatures which ranged from 22 to 29°.

Separation Rate Evaluation.—Preliminary work showed that water separation was the only visible change to occur in the emulsion. Consequently, a 100.0-ml. fraction of each emulsion sample was placed in a rubber stoppered Pyrex glass tube as outlined by Peck (12). Water separation was measured at frequent intervals for 180 days.

Particle Size Determination.—Immediately after manufacture, each sample was viewed with a standard optical microscope equipped with a 10× eyepiece and 43× objective. Emulsion samples were "mixed" prior to sampling by gently tumbling them in the sample container by hand. The sample was prepared by diluting one drop of emulsion as delivered by a standard U.S.P. medicine dropper with 5.0 ml. of distilled water. One drop of this dilution was then placed on a glass slide, a cover slip carefully put in place, and the sample viewed. The longest regular dimension of each particle was measured using a micrometer in the eyepiece.

The method of Harris, Horvitz, and Moon (13) was applied to preliminary particle size data obtained from two different emulsion samples to determine the number of particles required for a statistically valid sampling. With the test a maximum confidence interval of 0.5 μ, 99% confidence coefficient, 95% probability, with 99 degrees of freedom, a sample size of 300 to 400 particles was indicated. Subsequently, 400 particles were counted from each emulsion sample in the determination of particle size parameters.

RESULTS AND DISCUSSION

Rheology.—The temperature of emulsification and rate of cooling altered specific rheological properties of this emulsion system. All samples demonstrated plastic viscosity and thixotropy (Table II and Figs. 2–5). As shown by Table II, thixotropy increased greatly with age in all samples. The rate of cooling determined, to a large extent, the amount of thixotropy of the emulsion both initially and on aging.

[4] Keuffel and Essel Co., Model #4242.

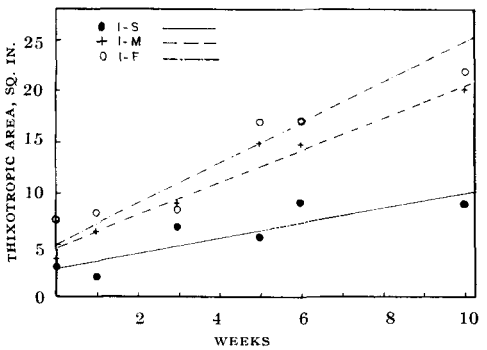


Fig. 3.—The effect of cooling rate and sample age on the thixotropy of a wax emulsion.

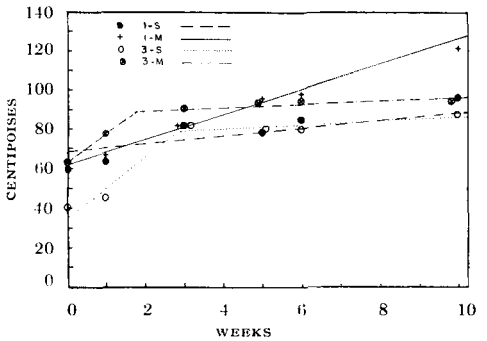


Fig. 4.—The effect of emulsification temperature, cooling rate, and sample age on the plastic viscosity of a wax emulsion.

The more rapid the cooling rate, the greater the thixotropy of the emulsion samples.

Emulsions made at different temperatures and cooled slowly showed, without exception, that the lower the temperature of emulsification, the greater the plastic viscosity, thixotropic area, and yield value of the freshly prepared samples (Tables I and II). After 10 weeks this was still true for the yield values. Compared with emulsions cooled at the fast and medium rates, the slowly cooled emulsions demonstrated the lowest values for plastic viscosity and thixotropic area (Table II).

As illustrated by Table II, emulsions prepared at different temperatures and cooled at a medium rate initially had only about a 10% difference in plastic viscosity. After 10 weeks there was a variance of about 40%. Similarly, the products cooled at a medium rate initially possessed nearly equal thixotropic areas; however, after 70 days there was a great percentage variance in the areas (Table II). Initially, the yield value of emulsion 1-M was greater than emulsions 2-M and 3-M (2-M and 3-M being nearly equal to each other). After 10 weeks there were only small differences in the yield values in emulsions 1-M, 2-M, and 3-M.

The results indicate that the rapidly cooled products possessed the greatest thixotropy and yield value of all emulsions.

Despite the frequently published statement that the separate emulsion phases should not be prepared at widely differing temperatures, with this emulsion it seemed to make little difference in any property measured whether the phase temperatures varied by as much as 20° or not.

TABLE III.—PARTICLE SIZE DATA

Emulsion	Diameter, μ (Arithmetic Mean)	Emulsion	Diameter, μ (Arithmetic Mean)
1-S	6.1	3-F	4.9[a]
1-M	5.3	4-S	5.7
1-F	5.0	4-M	5.0
2-S	7.0	4-F	4.5[a]
2-M	6.2	5-S	5.9
2-F	5.9[a]	5-M	4.6
3-S	6.3	5-F	3.9[a]
3-M	5.9		

[a] These emulsions contained, in addition to the single crystals, many 35 to 50 μ aggregates.

TABLE IV.—SUMMARY OF ANALYSIS OF VARIANCE

Source	*d.f.*	SS.	M.S.	$F_{calcd.}$	$F_{0.05}$	Significant
Among columns, (cooling rate)	2	4.6	2.3	30.7	4.46	Yes
Among rows, (emulsion temp.)	4	4.3	1.08	14.3	3.84	Yes
Error	8	0.6	0.075	..	..	
Total	14	9.5				

Figure 5 shows that rheological replication was possible with this emulsion system. Emulsions 4-S and 4-S' were made, on separate days, at the same temperature and cooled at the same rate.

Separation Rate.—All samples showed water separation. Fast cooled emulsions, with the exception of emulsion 1-F which was prepared at a temperature just above the melting point of white wax (70° both phases), showed rapid pronounced water separation (Fig. 6). After 180 days, 27 to 32 ml. of water had separated from the fast cooled emulsions; however, only 13 ml. of water had separated from emulsion 1-F during this period. Water separation in the samples cooled at the slow and moderate rates varied from 10 to 14 ml. after 180 days. This difference was apparently due to the fact that in the fast cooled emulsions the phases had considerably less time for complete emulsification and mixing (Table I). Emulsion samples cooled at a medium rate consistently showed the least water separation over the test period.

Particle Size.—The study of the particles and their relative sizes under the microscope was quite revealing (Table III). The average particle size was definitely related to the rate of cooling and temperature of emulsification. In the emulsion samples, the average particle size varied from 3.9 μ in emulsion 5-F to 7.0 μ in emulsion 2-S. Without exception, smaller particles resulted as the rate of cooling was increased. However, fast cooled samples also contained large (35 to 50 μ) aggregates in addition to the small individual particles. This was probably due to incomplete emulsification of the sample. It is important to point out that only a few isolated oil globules were seen under the microscope. The mineral oil and the white wax apparently merged, forming needle-like crystals. De Navarre (14) states that when reacting sodium

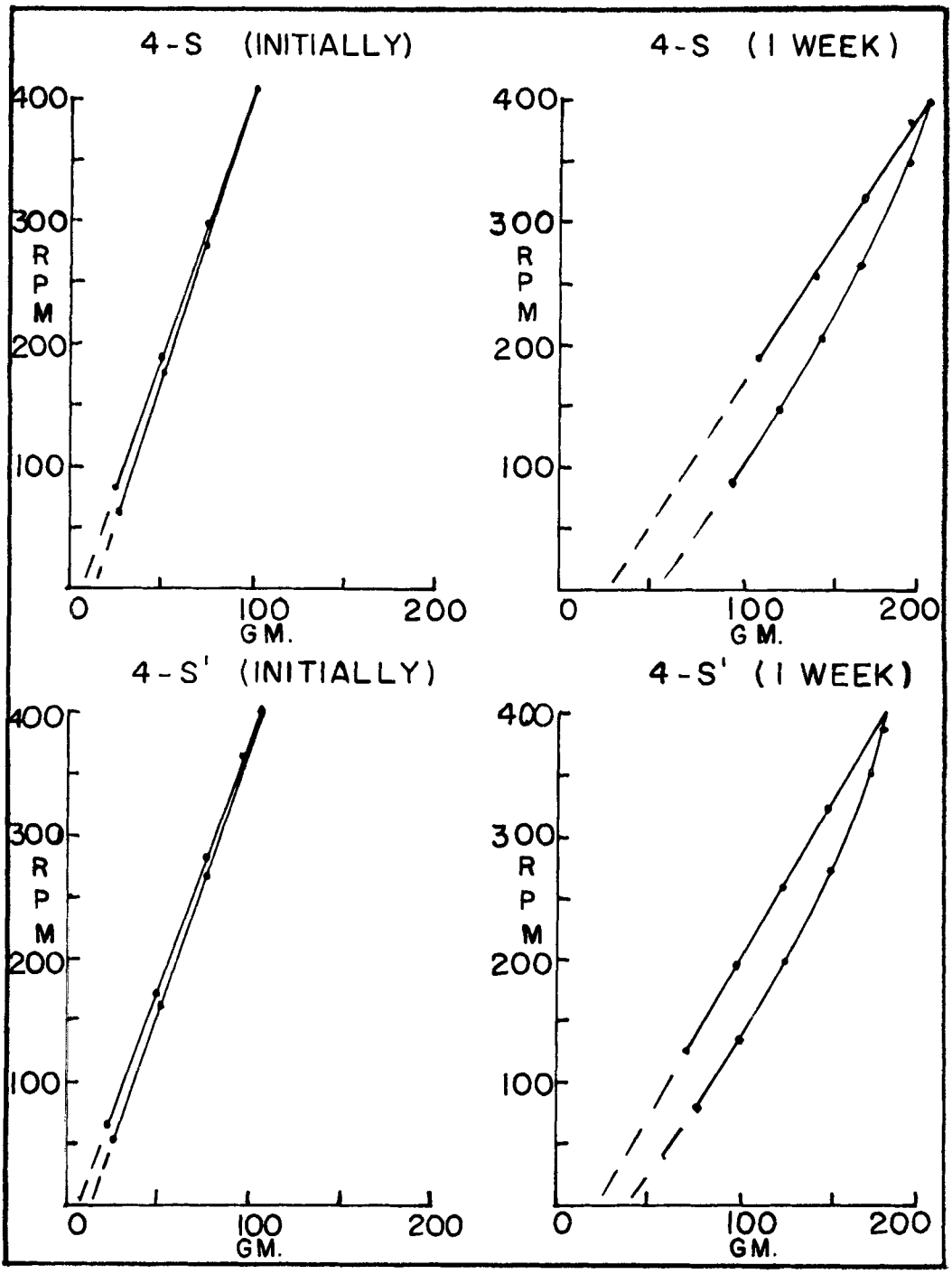


Fig. 5.—Rheological replication of emulsion 4-S.

borate with white wax to form a cold cream, sodium cerotate and several other minor soaps are formed which emulsify the oil and the waxes in the water. To determine if the oil and the wax would combine without the aid of an emulsifier, 80 Gm. of white wax and 250 Gm. of mineral oil were heated together, with mixing, to 80° and maintained at that temperature until all the wax melted. The mixture was then slowly cooled to room temperature. A uniform, semisolid, one-phase product resulted.

A two-way analysis of variance (15) revealed that both the rate of cooling and the temperature of emulsification produced significant changes in the average particle size (Table IV).

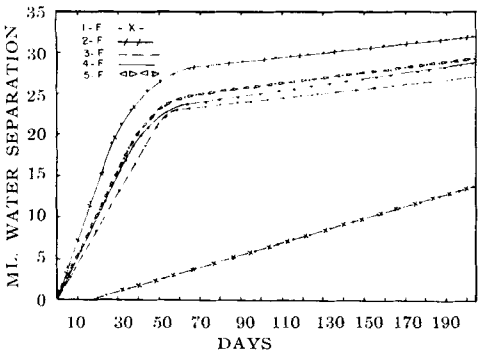


Fig. 6.—Water separation from fast cooled emulsions.

SUMMARY AND CONCLUSIONS

All the samples studied demonstrated plastic viscosity and thixotropy and possessed a yield value. However, the temperature of emulsification and rate of cooling definitely altered these and other physical properties of the emulsion. The rate of cooling was the main variable factor which determined the degree of thixotropy in the sample; the faster the cooling rate, the greater the thixotropy. As a general rule, plastic viscosity and thixotropy increased with age. Emulsions cooled at a medium rate showed more variance in their rheological values and trends than did the slowly cooled products.

Upon cooling, the wax and the mineral oil in the emulsion combined, forming needle-like crystals. Both the cooling rate and the emulsification temperature significantly changed the size of the emulsion particles; without exception, smaller particles resulted as the rate of cooling was increased. However, fast cooled emulsions formed wax-oil aggregates as well as individual particles.

All fast cooled emulsions, with the exception of the one prepared at 70° (both phases), showed rapid water separation over a 180-day interval. All other emulsions showed only slight water separation in the 180 day test period. Fast cooling is not recommended when preparing this emulsion.

Despite the frequently published statement that the separate emulsion phases should not be prepared at widely differing temperatures, with this emulsion it appeared to make little difference visually, microscopically, or rheologically whether the phase temperatures were the same or not.

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Direct Acidimetric Analysis of Papaverine in Concentrated Lithium Chloride Solution

By JAMES L. SPAHR, MODEST PERNAROWSKI, and ADELBERT M. KNEVEL

Hydrochloric acid was used as the titrant for papaverine base in two different media: (*a*) 18.5% ethanol and 81.5% 8 *M* lithium chloride solution, and (*b*) 10% tetrahydrofuran, 5% ethanol, and 85% 8 *M* lithium chloride solution. Per cents were calculated on a weight to weight basis. All of the titrations were carried out potentiometrically and the end points were determined by the first derivative method. It was found that bromophenol blue indicator could also be used to determine end points in medium *a*. The mean per cent recovery for the titrations in medium *a*, with the end points determined by the first derivative method, was 98.85 with a standard deviation of 0.69. In the same medium, using bromophenol blue for end point determination, the recovery was 99.48 with a standard deviation of 0.96. In medium *b*, the recovery was 100.02 with a standard deviation of 1.27.

CRITCHFIELD and Johnson (1) have found that the potentiometric break in the titration of a weak base with aqueous hydrochloric acid is greatly enhanced if a concentrated aqueous solution of a neutral salt is used as the titration medium. (Neutral salts, for the purpose of this work, are defined as those derived from strong monobasic inorganic acids and strong mono- or diacidic inorganic bases.) Aniline, for example, with an ionization constant of 3×10^{-10}, is too weak to be titrated satisfactorily in water. By titrating the same compound in 7 *M* sodium iodide solution, a potentiometric break is obtained which is satisfactory for precise analytical measurements.

The present study was undertaken with the purpose of investigating the possibility of analyzing certain pharmaceuticals by the above method. Many pharmaceutical compounds contain tertiary amine structures and can be classed as weak bases. It would appear, therefore, that they might lend themselves to this type of analysis.

Papaverine, which is representative of the type of compound to which this method of analysis may be applied, was selected as the base for this investigation. First, the compound had to have an ionization constant between 1×10^{-8} and 1×10^{-11} (1). It has been observed that compounds with ionization constants greater than 1×10^{-8} can be acidimetrically titrated very satisfactorily in water either by a direct or an indirect method. Compounds with ionization constants smaller than 1×10^{-11} cannot be titrated satisfactorily even in concentrated salt solutions.

Received May 23, 1961, from the School of Pharmacy, Purdue University, Lafayette, Ind.
Accepted for publication October 27, 1961.

Another reason for the selection of papaverine was that it was desired to choose a compound for which this method of analysis might be compared to the official procedure. The U.S.P. XVI method of analysis of papaverine hydrochloride preparations is a gravimetric one.

Lithium chloride was selected as the salt to be used in the titration medium because of its high heat of solution and because it has been successfully employed in titrations of this kind (1). Critchfield and Johnson have attempted to correlate the heat of solution of a salt with its effect on an acid solution. Their experimental data indicate that salts with high heats of solution exhibit the most pronounced effect of increasing the acidity of aqueous mineral acids. They have suggested that these salts have strong affinities for water and, therefore, compete with the hydronium ion for water of hydration (2). This, in effect, decreases the hydration of the hydronium ion and causes it to be more active.

The object of this investigation was to devise a direct acidimetric method of analysis of papaverine. The experimental work required to meet this objective was carried out in three main steps. The first was finding a cosolvent which would solubilize the papaverine in concentrated lithium chloride solution and not interfere with end point detection. The second step was establishing the accuracy and precision which could be obtained by this direct titration procedure. This was done by titrating known samples of papaverine and calculating per cent recovery. The third step was comparing the accuracy of the titration and the gravimetric methods. This was done by analyzing several official papaverine preparations by both methods and comparing the results.

EXPERIMENTAL

General Titration Procedure

All titrations were carried out potentiometrically using a Leeds and Northrup line-operated pH meter[1] equipped with glass and calomel electrodes. The titrant was added from a 5-ml. buret and the titration mixture was stirred throughout the process with Sargent magnetic stirrer.[2] The applicability of the potentiometric curves to end point determination was judged intuitively. Unless otherwise indicated, end points were determined by the first derivative method. That is, the volume at which the greatest value of dpH/dV occurred was taken as the end point.

Reagents

Hydrochloric acid, at a normality near 0.05, was used as the titrant. This acid was standardized against a 0.1 N sodium hydroxide solution which was standardarized using potassium acid phthalate. The lithium chloride was a Baker analyzed reagent.[3]

Preparation of Samples

Ion Exchange.—Papaverine hydrochloride was converted to the free base by means of an ion exchange resin. The procedure proposed by Levi and Farmilo (3) was used with the following alterations: Dowex 1-X4[4] resin was substituted for Amberlite IR-4B[5] and the sample size was reduced from 75 mg. to 65 mg. Individual samples were prepared by dissolving 0.6500 Gm. of papaverine hydrochloride in 100 ml. of the solution in a 100-ml. volumetric flask and pipetting 10-ml. aliquots. The solvent used in this step consisted of two parts by volume of chloroform and one part by volume of ethanol. The aliquots were introduced onto the ion exchange column and a solution of the free papaverine base was eluted. The solvent was removed by evaporation on a water bath, and the sample was then ready to be dissolved in the appropriate solvent and titrated with acid.

The columns were checked for completeness of recovery by processing a sample in the above manner, collecting the eluted papaverine solution in ten 10-ml. fractions, and testing these fractions for papaverine by the U.S.P. XVI identification test (4). Recovery was found to be essentially complete. Samples prepared in this way are referred to as column samples.

Extraction.—Papaverine was extracted from papaverine hydrochloride according to the method described in U.S.P. XVI under the assay of papaverine hydrochloride injection. Individual samples were prepared by weighing portions of the dried free base. Samples prepared in this way are referred to as extracted samples.

Solubilization of Papaverine in 8 *M* Lithium Chloride

The very low solubility of papaverine in water requires the use of cosolvents to produce a homogeneous system in 8 *M* lithium chloride solution.

Tetrahydrofuran-Ethanol (Alcohol U.S.P. XVI).—Tetrahydrofuran (THF) is a good solvent for papaverine. However, it is immiscible with 8 *M* lithium chloride and hence a third solvent, ethanol, is required to solubilize certain concentrations of THF in 8 *M* lithium chloride. These three comprise a typical ternary system, the compositions of which can be described graphically by means of a triangular plot. This plot was constructed by the following procedure: a series of seven solutions was prepared containing known amounts of ethanol and 8 *M* lithium chloride, and these were titrated with THF. The end point was taken as the first perceptible cloudiness. A second series of seven solutions was prepared containing known amounts of ethanol and THF. These were titrated to cloudiness with 8 *M* lithium chloride. The per cent composition of each sample at the end point was calculated on a weight basis and these compositions were plotted on triangular graph paper. This plot is shown in Fig. 1. At any point above the curved line, the system is homogeneous. At any point below the line, the system consists of two phases.

From the triangular plot, different compositions were selected with the objective of keeping the THF and ethanol concentration at the lowest possible value which would solubilize the papaverine and produce a satisfactory titration curve. The combination which gave the best results with respect to solubility and per cent recovery was 10% THF, 5% ethanol, and 85% 8 *M* lithium chloride solutions. A convenient volume was chosen to be 5.6 ml. of THF, 3.2 ml. of ethanol, and 36 ml. of 8 *M* lithium chloride. Ten column samples were analyzed using this system.

It was observed that when column samples were dissolved in this medium, the solution was slightly turbid. This cloudiness was caused by a small amount of insoluble residue eluted from the ion exchange resin.

It was found that ethanol alone would solubilize a column sample of papaverine. This solution also had a slightly turbid appearance which was again attributed to the presence of an insoluble residue eluted from the resin. However, when the first

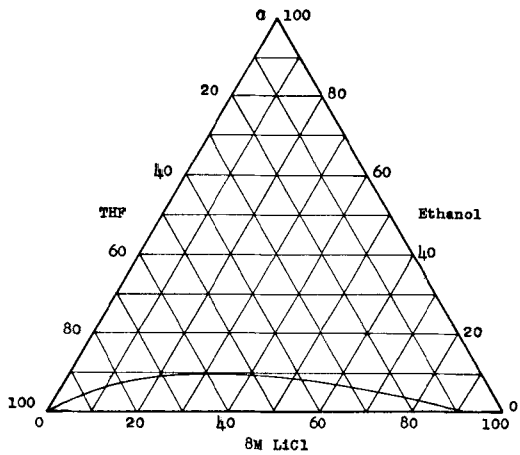


Fig. 1.—Three-component system consisting of tetrahydrofuran, ethanol, and 8 *M* lithium chloride solution.

[1] Leeds and Northrup Co., Philadelphia, Pa.
[2] E. H. Sargent Co., Chicago, Ill.
[3] J. T. Baker Co., Phillipsburg, N. J.
[4] Dow Chemical Co., Fair Lawn, N. J.
[5] Fisher Scientific Co., Fair Lawn, N. J.

increment of acid was added, a small amount of precipitate formed. This did not occur when the three-component system was used as the titration medium. The precipitate was slight and did not interfere with the end point determination. It was not identified.

Ethanol.—Ethanol also proved to be a good co-solvent for extracted samples. A system consisting of 18.5% (12 ml.) of ethanol and 81.5% (36 ml.) of 8 *M* lithium chloride solution gave good results with 5 samples. Volumes of ethanol less than 12 ml. failed to keep the papaverine in solution. Volumes greater than 15 ml. tended to flatten the curve and cause erratic pH meter response.

Figure 2 shows curves obtained in the titration of extracted samples. Curve 1 is the titration in the ethanol-8 *M* lithium chloride system and curve 2 is the titration with the same amount of ethanol but with the 8 *M* lithium chloride solution replaced by water.

It was found that bromophenol blue indicator gave satisfactory end points in this system. The color change is from blue to faint yellow.

For the solubilization of a column sample, 15 ml. of ethanol was required. The need for this increased volume might possibly have been due to the presence of the insoluble column residue. At this concentration of ethanol, the potentiometric curve was leveled somewhat and the end point was not as clearly indicated. Therefore, the tertiary mixture was used as the titration medium of choice for column samples. It was observed, when calculating the per cent recovery of a series of samples, that the results were consistently high. This suggested that the medium was contributing a certain amount of basicity. A series of titrations performed on the components of the system indicated that the lithium chloride was the source of error and demanded that a blank value be determined. This blank value was not strictly reproducible by direct titration of the lithium chloride solution with acid so a back titration procedure was tried. Standard acid was added to the lithium chloride solution and the excess was titrated with sodium hydroxide. The milliequivalents of acid equivalent to the basicity of 36 ml. of 8 *M* lithium chloride solution was calculated. Reproducible results were obtained by this procedure and it was accepted as a satisfactory method for the determination of the blank value. A certain amount of variation can be observed in the blank values recorded in Tables I, II, and III. This is accounted for by the fact that the 8 *M* lithium chloride solution was only approximate and, therefore, a new blank value had to be determined for each batch of solution which was prepared. Another reason for the variation is that all the lithium chloride used did not come from the same lot.

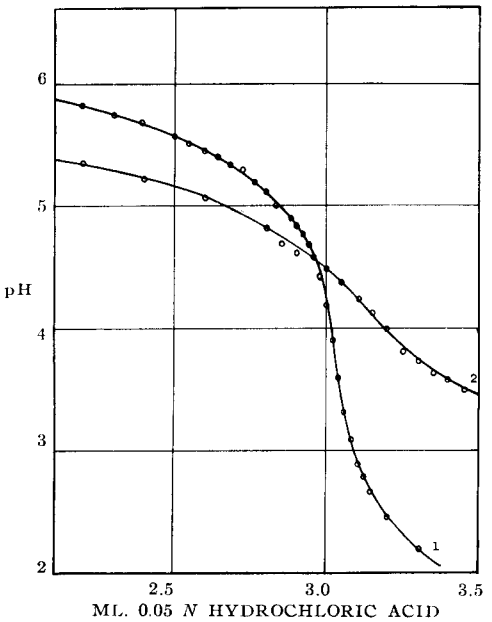


Fig. 2.—Potentiometric titration of papaverine. 1, In 18.5% ethanol and 81.5% 8 *M* lithium chloride solution; 2, In 18.5% ethanol and 81.5% water.

TABLE I.—POTENTIOMETRIC TITRATION OF PAPAVERINE IN 10% TETRAHYDROFURAN, 5% ETHANOL, AND 85% 8 *M* LITHIUM CHLORIDE

Sample No.	Sample Wt., Gm.	Acid Used, meq.	Blank, meq.	% Recovery
1	0.0587	0.1896	0.0188	98.75
2	0.0587	0.1933	0.0188	101.54
3	0.0587	0.1896	0.0188	98.75
4	0.0587	0.1933	0.0188	101.54
5	0.0587	0.1933	0.0188	101.54
6	0.0587	0.1834	0.0092	100.71
7	0.0587	0.1964	0.0233	100.09
8	0.0587	0.1934	0.0233	98.35
9	0.0587	0.1907	0.0198	98.83
10	0.0587	0.1929	0.0198	100.08
			Mean	100.02
			Standard Deviation	1.27

TABLE II.—POTENTIOMETRIC TITRATION OF PAPAVERINE IN 18.5% ETHANOL AND 81.5% *M* LITHIUM CHLORIDE SOLUTION

Sample No.	Sample Wt., Gm.	Acid Used, meq.	Blank, meq.	% Recovery
1	0.0581	0.1977	0.0276	99.36
2	0.0598	0.2002	0.0276	97.90
3	0.0589	0.1997	0.0276	99.13
4	0.0514	0.1783	0.0276	99.50
5	0.0590	0.1986	0.0276	98.36
			Mean	98.85
			Standard Deviation	0.69

TABLE III.—POTENTIOMETRIC TITRATION OF PAPAVERINE IN 18.5% ETHANOL AND 81.5% 8 *M* LITHIUM CHLORIDE SOLUTION

Sample No.	Sample Wt., Gm.	Acid Used, meq.	Blank, meq.	% Recovery
1	0.0581	0.1977	0.0276	99.36
2	0.0598	0.2008	0.0276	97.90
3	0.0598	0.2008	0.0276	100.20
4	0.0514	0.1794	0.0276	100.19
5	0.0590	0.2018	0.0276	100.19
			Mean	99.48
			Standard Deviation	0.96

TABLE IV.—RESULTS OF THE ANALYSIS OF PAPAVERINE HYDROCHLORIDE TABLETS (0.0324 GM./TAB.) BY GRAVIMETRIC AND TITRIMETRIC METHODS

Sample No.	Papaverine HCl Present,[a] Gm.	Gravimetrically		Titration	
		Found, Gm.	Recovery, %	Found, Gm.	Recovery, %
1	0.1022	0.1014	99.22	0.1017	99.49
2	0.1022	0.1046	102.36	0.1009	98.73
3	0.1026	0.1020	99.33	0.1021	99.45
4	0.1026	0.0989	96.31	0.1009	98.35
		Mean	99.31	Mean	99.00
		Standard Deviation	2.47	Standard Deviation	0.52

[a] Calculated from the labeled amount.

TABLE V.—RESULTS OF THE ANALYSIS OF PAPAVERINE HYDROCHLORIDE INJECTION (0.0320 GM./ML.) BY GRAVIMETRIC AND TITRIMETRIC METHODS

Sample No.	Papaverine HCl Present,[a] Gm.	Gravimetrically		Titration	
		Found, Gm.	Recovery, %	Found, Gm.	Recovery, %
1	0.128	0.1342	104.82	0.1274	99.51
2	0.128	0.1357	106.30	0.1321	103.16
3	0.128	0.1317	102.91	0.1297	101.34
4	0.128	0.1332	104.04	0.1305	101.95
		Mean	104.52	Mean	101.49
		Standard Deviation	1.01	Standard Deviation	1.15

[a] Calculated from the labeled amount.

Assay of Some Official Papaverine Preparations

Papaverine hydrochloride injection[6] (0.032 Gm./ml.) and papaverine hydrochloride tablets[6] (0.0324 Gm./tab.) were assayed according to the U.S.P. XVI procedures. After the samples were dried and weighed, they were dissolved in 20 ml. (18.5% w/w) of ethanol and 60 ml. (81.5% w/w) of 8 *M* lithium chloride solution and titrated with 0.1 *N* hydrochloric acid. Either titration medium previously outlined could have been used. The ethanol-THF combination will solubilize a greater amount of papaverine per unit volume than will ethanol alone. However, the preparation of the three-component medium requires one additional volume measurement, i.e., THF. Since both systems give satisfactory curves with extracted samples, the ethanol-8 *M* lithium chloride medium was selected because of its relative ease of preparation.

RESULTS

Table I lists the per cent recovery for 10 column samples of papaverine titrated in 10% THF, 5% ethanol, and 85% 8 *M* lithium chloride solution. End points were determined by the first derivative method. The mean per cent recovery indicates good accuracy. The deviation of 1.27% represents about 0.05 ml. of 0.05 *N* acid, about 0.002 meq., and about 0.7 mg. of papaverine.

Table II lists the per cent recovery obtained from the five extracted samples titrated in 18.5% ethanol and 81.5% 8 *M* lithium chloride solution. In this table the end points were determined by the first derivative method. Table III lists the recovery of the same five samples, but the end points were determined with bromophenol blue indicator. The alkaline form of bromophenol blue is bluish-purple in this system. The end point occurred at pH 2.8–3.5 and the color changed to an easily perceptible faint bluish-yellow. The change is abrupt enough to be used in routine analysis.

[6] Eli Lilly and Co., Indianapolis, Ind.

Tables IV and V list the per cent recovery obtained in the assay of two official preparations. End points for the titrimetric data were determined by the first derivative method. These same end points were also determined using bromophenol blue and the results obtained were essentially equivalent to those by the first derivative. Since the exact amount of papaverine in the samples is unknown, the mean per cent recovery is not as significant as that of the known samples. The standard deviation is, however, a valid measure of precision.

CONCLUSIONS

The data obtained in this investigation indicate that this method of analysis is suitable for the quantitative determination of papaverine and other alkaloids or pharmaceuticals whose properties are similar to those of papaverine.

The problem of solubilizing the free base was overcome by using cosolvents in either a binary or ternary system. The optimum volume of the cosolvent was that volume which would hold the papaverine in solution in 8 *M* lithium chloride and not cause excessive dilution. This investigation indicates that 8 *M* lithium chloride solution can be diluted 33% with cosolvent and still sufficiently enhance the potentiometric break when titrating papaverine.

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Chlorpromazine Hyperthermia in Young Albino Mice

By WALTER J. BAGDON and DAVID E. MANN, Jr.

The intraperitoneal administration of chlorpromazine hydrochloride to albino mice, ranging in age from 10 to 38 days, caused hyperthermia in the younger and hypothermia in the older animals. No significant difference due to sex was observed in the effect of the drug at different ages.

THE BODY temperature of the homoiothermic vertebrate, contrary to the poikilothermic animal, is controlled within narrow limits by hypothalamic coordinating centers which maintain a delicate balance between heat production and dissipation, and function independently of environmental thermal changes (1). In 1951, during the preliminary pharmacologic investigations of the phenothiazine tranquilizers, it was observed by Courvoisier and her co-workers (2) that chlorpromazine could temporarily transform an homoiotherm into a poikilotherm, an effect later denied by Decourt, *et al.* (3). Later that year, despite this controversy, Laborit and Huguenard (4) introduced chlorpromazine into clinical medicine as a preoperative drug in conjunction with promethazine and meperidine (the lytic cocktail), a combination that caused a fall in body temperature with the attainment of a state of "artificial hibernation," presumably conducive to the prevention of postoperative shock. The hypothermic action of chlorpromazine in a wide variety of mature animals, including mice (1, 5–7), is now well established, and many factors have been found which affect this response (6, 8–13), yet a paucity of information exists in the literature with regard to the effect of this agent on the normal body temperature of immature animals. Therefore, it was the purpose of this study to observe the effects of chlorpromazine when administered to young mice of various ages.

EXPERIMENTAL

Albino mice (1321) (Huntingdon Farms, Inc., HTF strain) were divided into nine groups, each containing from 116 to 238 animals, according to age as follows: 10, 15, 20, 25, 27, 30, 31, 35, and 38 days old. The variation in age of mice within a group did not vary more than 15 hours. With the exception of the youngest age groups (10 and 15 days), each group was equally divided as to sex. Food was withdrawn 15 minutes and water 1 hour before experimentation to provide for nutritional constancy. Temperature recordings, to the nearest 0.1°, were determined orally immediately prior to and 30 minutes after each injection with a model 43 Tele-Thermometer equipped with a No. 402 probe.[1] During testing, each animal was confined in a 100-ml. glass beaker with a screen top, and kept at a constant environmental temperature of 38° 15 minutes before injection and throughout the 30-minute observation period. Freshly prepared aqueous solutions of chlorpromazine hydrochloride[2] were administered at a dosage level of 1 mg./Kg., a dose known to produce hypothermia in mature mice (5, 7), to one-half of a group (equally divided as to sex), while the remaining mice received distilled water and served as controls. All injections were given intraperitoneally at a fixed volume of 0.1 ml.

RESULTS AND DISCUSSION

The effect of chlorpromazine hydrochloride, administered intraperitoneally at a dosage level of 1 mg./Kg., on the body temperature of young albino mice was shown to be influenced by age as recorded in Table I. From the data obtained from 1321 animals, the differences between "before" and "after" temperatures and means for each group were calculated. The significance of differences between means was estimated by use of the *t* test and results are shown in Table II. Only probability levels of 5% or below are indicated. It can be seen that the differences between drug and control groups are significant at extremes of age only, and that there is no evidence that the differences at ages of 20 through 30 days could not have occurred by chance.

Differences between male and female groups at every age are small, and it could be similarly shown that they do not reach levels of statistical significance.

Even if the male and female groups are combined for ages 20 through 31 days, as in Table III, only the 30 and 31-day-old groups reach the 5% level of probability; thus, there is no evidence of either hyperthermia or hypothermia at the ages of 20, 25, or 27 days.

In order to test the significance of the drug × age interaction, the male and female groups were combined and tables of random numbers were used to discard three cases from the 25-day-control group, two from the 27-day-control group, two from the 30-day-drug group, and two from the 38-day-control group, leaving numbers in groups as shown in Table IV. An analysis of variance was then performed as shown in Table V.

Received April 28, 1961, from the School of Pharmacy, Temple University, Philadelphia 40, Pa.

Accepted for publication November 6, 1961.

Abstracted from a thesis presented to the Graduate School of Temple University, School of Pharmacy, by Walter J. Bagdon in partial fulfillment of the requirements for the degree of Master of Science.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

The authors are indebted to Dr. A. A. Sugerman for the statistical analysis and to Dr. Neil Chilton for his technical advice.

[1] Yellow Springs Instrument Co., Yellow Springs, Ohio.

[2] Marketed as Thorazine by Smith Kline & French Laboratories, Philadelphia 1, Pa.

TABLE I.—EFFECT OF CHLORPROMAZINE HYDROCHLORIDE ON THE BODY TEMPERATURE OF YOUNG ALBINO MICE[a]

	No. of Mice	Sex	Wt., Gm.	Temperature, °C. Initial	Final	Change	Chlorpromazine Change
10-Day-old							
Drug	106	..	4.73	35.94	37.42	+1.48	+0.91
Control	106	..	4.79	35.92	36.49	+0.57	
15-Day-old							
Drug	60	..	7.27	37.12	37.54	+0.42	+0.74
Control	60	..	7.23	37.27	36.95	−0.32	
20-Day-old							
Drug	28	M	9.27	37.19	37.24	+0.05	+0.14
Control	30	M	9.97	37.08	36.99	−0.09	
Drug	30	F	9.24	37.63	37.66	+0.03	+0.01
Control	28	F	9.78	36.81	36.83	+0.02	
25-Day-old							
Drug	35	M	12.32	37.67	37.51	−0.16	−0.12
Control	34	M	12.39	37.50	37.46	−0.04	
Drug	33	F	12.47	37.69	37.57	−0.12	0.00
Control	37	F	12.17	37.53	37.41	−0.12	
27-Day-old							
Drug	29	M	12.78	37.72	37.64	−0.08	−0.07
Control	30	M	12.35	37.43	37.42	−0.01	
Drug	29	F	12.10	37.71	37.65	−0.06	0.00
Control	30	F	12.13	37.50	37.44	−0.06	
30-Day-old							
Drug	61	M	16.48	37.38	37.16	−0.22	−0.22
Control	58	M	15.57	37.46	37.46	0.00	
Drug	59	F	15.48	37.40	37.19	−0.21	−0.13
Control	60	F	15.45	37.75	37.67	−0.08	
31-Day-old							
Drug	30	M	17.73	36.93	36.57	−0.36	−0.32
Control	31	M	17.53	36.99	36.95	−0.04	
Drug	29	F	17.40	37.16	36.89	−0.27	−0.25
Control	28	F	17.35	37.18	37.16	−0.02	
35-Day-old							
Drug	29	M	20.44	37.01	36.48	−0.53	−0.50
Control	29	M	20.06	36.46	36.43	−0.03	
Drug	30	F	18.54	36.87	36.49	−0.38	−0.37
Control	30	F	18.48	36.47	36.46	−0.01	
38-Day-old							
Drug	34	M	19.81	36.74	36.10	−0.64	−0.62
Control	34	M	20.22	36.78	36.76	−0.02	
Drug	36	F	18.80	36.48	36.06	−0.42	−0.40
Control	38	F	18.54	36.67	36.65	−0.02	

[a] Drug administered intraperitoneally, 1 mg./Kg.

TABLE II.—SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS FOR EACH GROUP, ESTIMATED BY THE *t* TEST

Age, day	Sex	Drug *N*	Drug Mean	Control *N*	Control Mean	Diff.	*t*	*P*
10	..	106	1.485	106	0.574	0.911	6.55	0.001
15	..	60	0.418	60	−0.318	0.736	5.14	0.001
20	M	28	0.054	30	−0.090	0.144	0.88	[a]
20	F	30	0.033	28	0.021	0.012	0.06	[a]
25	M	35	−0.154	34	−0.035	−0.119	0.82	[a]
25	F	33	−0.118	37	−0.119	0.001	0.001	[a]
27	M	29	−0.079	30	−0.003	−0.076	0.43	[a]
27	F	29	−0.062	30	−0.060	−0.002	0.01	[a]
30	M	61	−0.220	58	−0.000	−0.220	1.86	[a]
30	F	59	−0.215	60	−0.078	−0.137	1.09	[a]
31	M	30	−0.357	31	−0.039	−0.318	2.33	0.05
31	F	29	−0.269	28	−0.011	−0.258	1.78	[a]
35	M	29	−0.531	29	−0.021	−0.510	2.58	0.02
35	F	30	−0.373	30	−0.013	−0.360	2.11	0.05
38	M	34	−0.638	34	−0.024	−0.614	4.05	0.001
38	F	36	−0.425	38	−0.021	−0.404	3.07	0.01

[a] No significance.

It is seen (Table V) that the drug × age interaction is significant; i.e., the effect of the drug is different as age increases, and by inspecting the table of differences (Table II), it is seen that this effect is to increase temperature in the lower age groups and to reduce it in the higher age groups.

The significant main effect of age shown in Table V is of much less interest; by inspection of Table II

TABLE III.—SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS FOR COMBINED GROUPS, ESTIMATED BY THE *t* TEST

Age, day	Drug		Control		Diff.	*t*	*P*
	N	Mean	*N*	Mean			
20	58	0.043	58	−0.036	0.079	0.65	[a]
25	68	−0.137	71	−0.079	−0.058	0.57	[a]
27	58	−0.071	60	−0.032	−0.039	0.27	[a]
30	120	−0.218	118	−0.039	−0.179	2.07	0.05
31	59	−0.314	59	−0.027	−0.287	2.89	0.01

[a] No significance.

TABLE IV.—MODIFIED CONTROL GROUPS

Age, day	Drug	Control
10	106	106
15	60	60
20	58	58
25	68	68
27	58	58
30	118	118
31	59	59
35	59	59
38	70	70
Total N = 1312		

we can interpret this as reflecting the increasing hypothermia with age shown in the control groups as well as in the chlorpromazine groups.

To test for the presence of a significant drug × age × sex interaction, the two lowest age groups, undifferentiated as to sex, were discarded, and, as before, cases were discarded from the remaining groups using a table of random numbers, leaving numbers in groups as shown in Table VI. An analysis of variance was then performed which showed no significant age × sex × drug interaction, no significant sex × drug or sex × age interaction, or main effect of sex (*F* values, respectively, 0.29, 1.41, 0.17, and 0.24). Thus, there is no evidence that the change in effect of drug with age is different as between males and females.

SUMMARY

1. The data show that the effect of chlorpromazine on body temperature is influenced by age, the drug causing significant hyperthermia in 10 and 15-day-old mice and hypothermia in 35 and 38-day-old mice.

2. There is no significant evidence of a difference in the effect of chlorpromazine at various ages due to sex.

TABLE V.—ANALYSIS OF VARIANCE FOR TABLE IV DATA

Source of Variation	*df*	*MS*	*F*	*P*
Drug	1	1.16	2.19	not signif.
Age	8	30.72	57.96	0.001
Drug × age	8	10.41	19.64	0.001
Within cells	1294	0.53		
Total	1311			

TABLE VI.—GROUPS USED TO TEST FOR SIGNIFICANT DRUG × AGE × SEX INTERACTION

Age, day	Drug		Control	
	Male	Female	Male	Female
20	28	28	28	28
25	33	33	33	33
27	29	29	29	29
30	58	58	58	58
31	28	28	28	28
35	29	29	29	29
38	34	34	34	34
Total N = 956				

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Identification and Evaluation of Botanical Extracts by Paper Chromatography

By WILLIAM A. MEER†, ROLF S. WESTBY‡, and WALTER SIRGO†

The problem of identification of botanical drug extracts is enormous in view of the large variety of botanical species as well as other factors affecting the biosynthesis of plant constituents. This is particularly true for those drugs which do not have a pharmacologically active ingredient or chemical component which can be assayed for in a routine manner. A rapid method is proposed which utilizes an ascending paper chromatographic technique with slight modification, ultraviolet light for observation, and the use of appropriate chromogenic spray reagents. This procedure produces a chromatogram consisting of base, stem, cone, and solvent front regions. The resulting characteristic picture, when viewed as a whole in comparison with a standard or control sample, can then be used for the identification and evaluation of the drug extract.

THE IDENTIFICATION of botanical drugs is accomplished by a variety of analytical and biochemical methods. Generally speaking, these have been based on both macroscopic and microscopic pharmacognostical descriptions, color reactions, assay procedures for the determination of chemical constituents as well as specific physiological responses observed through biological assay techniques.

Due to the complexity of most drugs, these procedures are generally lengthy and rather intricate so that identification often is based solely on physical characteristics. In the case of extracts of these drugs, so often indistinguishable in such a form, identification poses an even greater problem. As many extracts, fluidextracts, tinctures, and similar preparations are daily analyzed in our laboratories, our concern was more immediate and led us to devise a rapid identification method. The natural choice was paper chromatography. The method described using this technique has met with good success and is now being used as a routine control procedure.

Accounts of the history of chromatography dating to about 1903 have been adequately covered by Lederer and Lederer (1), by Consden, Gordon, and Martin (2), and others.

Many techniques and adaptations of methods have widely increased the scope of chromatography. The use of ultraviolet light, suggested by the Portuguese scientist Konig in 1935 (3) to help detect or locate fluorescent substances, has been of great value. In a recent study by Kokoski, *et al.* (4), 133 powdered crude drugs were examined under ultraviolet light and the color of the fluorescence proposed for identification. A disadvantage noted was that there was some variation in color reported for the same crude drug by different observers.

Work supported jointly by the Vanilla Bean Association and the Flavoring Extract Manufacturers Association resulted in the development of a paper chromatographic method for the evaluation of vanilla extracts and the detection of foreign materials used for adulteration (5). An important and interesting result of this work has also been the development of a method whereby fluorescent chromatograms can be photographed in their true colors (6).

At present, the N.F. XI utilizes paper chromatography in the chemical identification test appearing in the monograph on *Rauwolfia serpentina* (7). The alkaloidal content of the sample is compared to a reference standard.

The problem of positive identification of botanical drug extracts is enormous in view of the large variety of botanical species as well as factors affecting the biosynthesis of plant constituents. This is particularly true for those drugs which do not have a pharmacologically active ingredient or chemical component which can be assayed for in a routine manner.

With all of these problems in mind, a rapid method is proposed which uses an ascending paper chromatographic technique with slight modification, ultraviolet light for observation, and, in some cases, the use of appropriate chromogenic spray reagents. This procedure allows for the examination of the drug extract as a whole. The amount of the extract used represents a very small amount of whole crude drug so that identification can be made with only a limited amount of material, the same amount of which may prove difficult or impossible by

Received July 24, 1961, from the Quality Control Laboratories, Meer Corp., New York, N. Y., and Eli Lilly and Co., Indianapolis, Ind.
Accepted for publication October 16, 1961.
† Meer Corp.
‡ Eli Lilly and Co.
The authors wish to thank Mr. W. E. Kruse for the art and photographic work.

microscopic examination. The resulting chromatogram will produce a characteristic picture and, when compared to a standard or control sample, can then be used for identification.

EXPERIMENTAL

Materials.—Fluid extracts (1 ml. = 1 Gm. crude drug). Tinctures must be concentrated from 0.1–0.2:1 strength to 1:1 ratio. Powdered extracts are reconstituted with the appropriate hydroalcoholic menstruum and filtered or centrifuged to remove insoluble solids (starch, marc, etc.). Solid or pilular extracts are appropriately reconstituted with menstruum to a 1:1 ratio.

Apparatus and Reagents.—A chromatographic chamber whose size will vary with the number of strips run; this may be a box constructed from cardboard, wood, or glass with a removable front and no top; this chamber helps to maintain an atmosphere somewhat saturated with the solvent while protecting the strips from air currents. Crystallizing dishes, 70 mm. in diameter and 50 mm. in depth. Graduated cylinders. Pipet to deliver 0.02 ml. Metal or glass rods and framework to support paper strips. Ventilating hood or similar exhaust system. Ultraviolet lamp with filter passing only long rays (3600–3700 Å.). Filter paper, Whatman No. 4, chromatographic grade, strips cut 2 × 11 in. with the paper fibers running lengthwise. Solvents: *n*-butyl alcohol, glacial acetic acid, distilled water (4:1:5). Chromogenic reagents to be sprayed on the developed and dried chromatographic strip.

Filter Paper Chromatographic Strip.—The paper strip is developed by ascending chromatography. Normal development time, useful for routine identification, requires 2 hours. Longer periods of time did not give improved resolution. Spots A and B represent points of application of the extract. Spot B is a reference spot and is useful to compare the concentration of one extract with another. After development of spot A, the chromatographic picture consists of the base, stem, cone, and solvent front regions. An area above the solvent front region sometimes becomes visible after the chromatogram is treated with an appropriate chromogenic reagent. A drawing representing a typical developed paper strip is shown in Fig. 1.

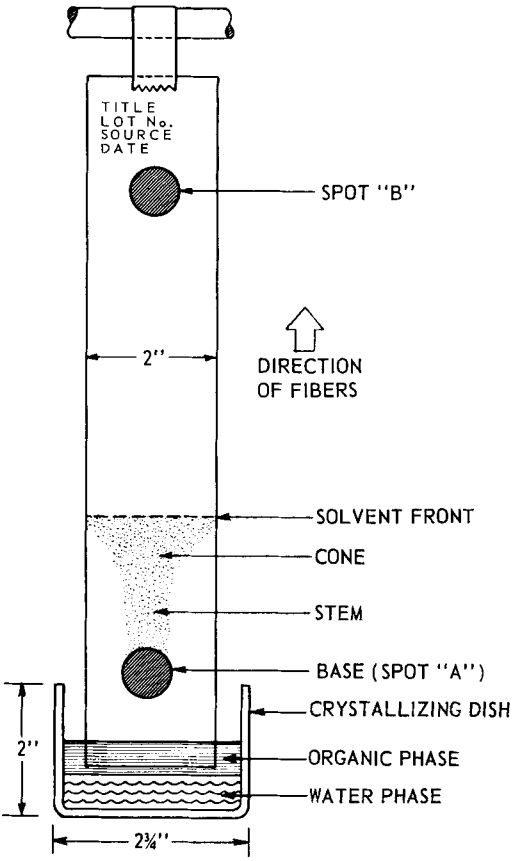


Fig. 1.—A drawing representing a typical developed paper strip.

Procedure.—Prepare the filter paper strip with the title of the extract, lot number, source, date, etc. With a pin and using a guide, mark the exact spots to apply the extract. Spot A centered 1 in. from the bottom edge of the strip and spot B centered 2 in. from the top edge of the strip. Place exactly 0.02 ml. of the extract on spot A and B and allow to dry, about 5 minutes. The extract should be applied slowly to keep the spots as small as possible. Use fluid extract strength (1 ml. = 1 Gm. crude drug). Prepare the solvent consisting of *n*-butyl alcohol, glacial acetic acid, distilled water (40:10:50), and shake vigorously in a separator (stopcock free from grease) for at least 1 minute to saturate before transferring the entire mixture to a crystallizing dish. The solvent is ready to use when the two layers are clear. The solvents should be freshly prepared and of the highest chromatographic grade. Immerse the paper strip in the organic phase so that the bottom edge of the strip is 2–3 mm. above the aqueous layer. Fasten the strips with scotch tape or clamps to a support rod about 12 in. above the bench top. Keep the temperature as constant as possible in the range of 20–25°. For checking reproducibility and comparison, run a reference standard or control and the unknown simultaneously. Each extract should be run in triplicate, not only to gain experience and to confirm reproducibility within each set, but to have additional strips for reagent treatment. Allow the chromatograms to develop for 2 hours with controllable ventilation. It is important to prevent drafts over the paper. Figure 2 shows three sets of chromatograms at the end of a 2-hour developing period. Dry the developed strips in a ventilated hood for 1 hour. The chromatograms should be viewed and evaluated soon after drying because some colors may change or disappear.

Results.—The developed and dried chromatograms are first viewed under visual and then ultraviolet light and the samples compared with the control or standard. Spot B should not show a variation of more than approximately ±10% in intensity. If the intensity varies greatly between the sample and the control, then the extract in question should be either diluted or concentrated to match more nearly the reference standard for identification purposes. Differences in intensity of spot B provide further information in the evaluation

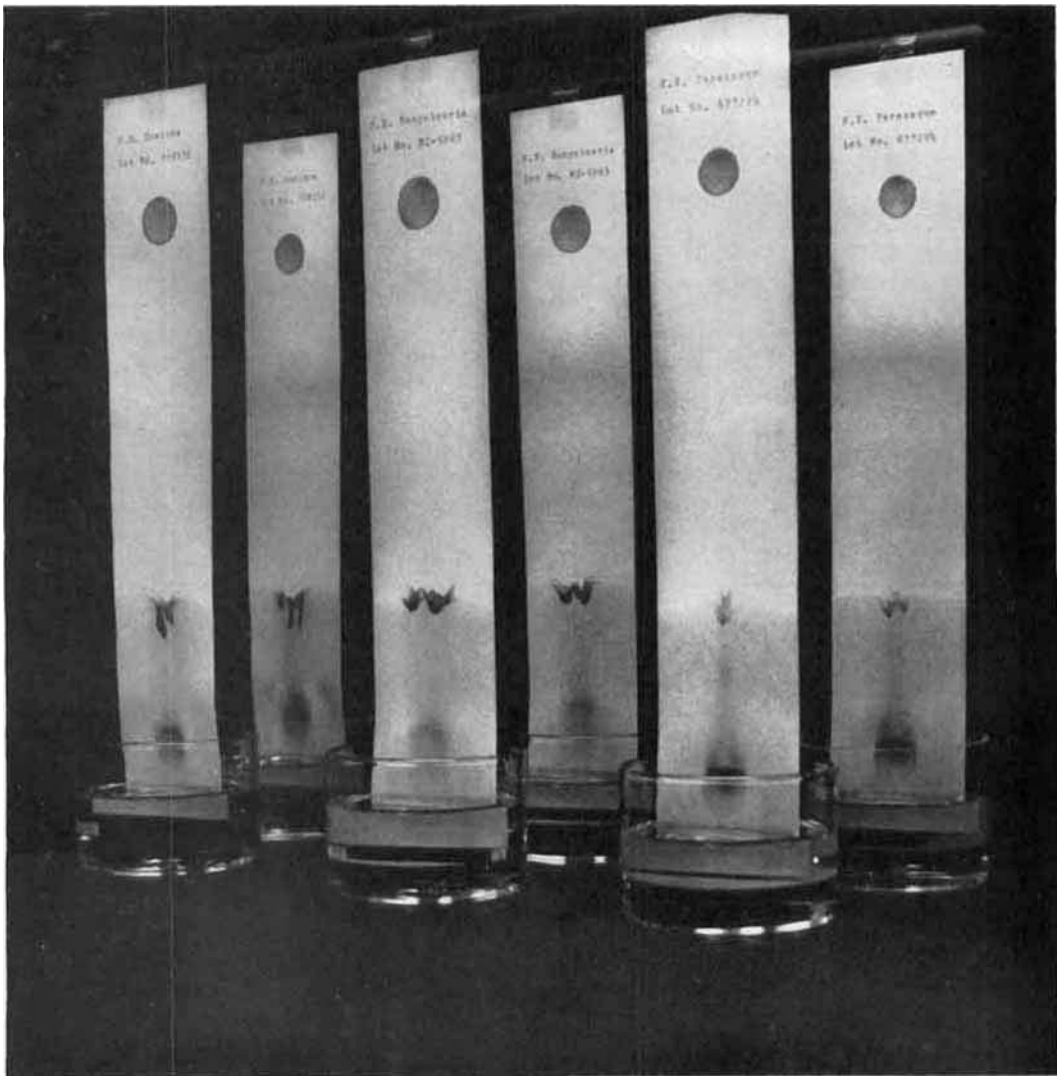


Fig. 2.—Three sets of chromatograms at the end of a 2-hour developing period. For this photograph, the protective side has been removed.

of the sample. A spot of lesser intensity generally indicates a lower concentration of soluble solids in the extract. Spot A has now traveled to form the base, stem, cone, and solvent front regions. Color comparisons should be made of these areas. Shade and intensity should be noted both under visible and ultraviolet light. The degree of travel, distribution, fluorescence, and relative position of the solute must also be observed.

Results have shown that a characteristic picture is produced for each extract tested (Fig. 2). A sample will therefore be positively identified when its developed chromatogram corresponds to that of the control after it has been subjected to the requirements described in the above procedure.

SUMMARY

This technique has proved to be a rapid analytical tool in the routine control, identification, and evaluation of botanical extracts. It produces a chromatogram consisting of base, stem, cone, and solvent front regions. By observing the chromatographic picture as a whole, an unknown can be determined when it matches a control or known sample by producing a total effect similar in distribution, intensity, coloring, and fluorescence. It must be remembered, however, that the variation in details may be due to the natural variation of botanical constituents.

If similar results are obtained with original chromatograms of extracts of different botanicals, further treatment with a chromogenic reagent may provide distinguishing characteristics. Preliminary work has indicated that it is difficult to ascertain the best chemical for use, especially due to the variety of compounds occurring in a

single extract. Effective use has been made of several reagents, however, such as: (*a*) a 0.1 *M* solution of cerium sulfate in water, (*b*) saturated methanol solution of aluminum sulfate, and (*c*) saturated water solution of sodium borate.

The common alkaloidal, sugar, and protein color producing reagents may also be employed. Work is in progress on the tabulation of extracts with corresponding spray reagents and will be described in a subsequent communication.

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Effects of Buffered and Unbuffered Acetylsalicylic Acid upon the Gastric Acidity of Normal Human Subjects

By CLARKE DAVISON, BENJAMIN W. SMITH, and PAUL K. SMITH†

Acetylsalicylic acid in tablet form, in buffered tablets, and effervescent buffered solution, has been compared with regard to the effect upon gastric acidity and pH. Aspirin and buffered aspirin have little or no effect as compared to controls, whereas the effervescent preparation reduces free acidity and raises gastric pH significantly for 30 minutes. This elevation of gastric pH correlates well with the more rapid absorption and reduced gastric irritation which has been reported for various soluble and buffered forms of salicylates.

DISADVANTAGES of acetylsalicylic acid as an analgetic and antipyretic agent are low solubility, which delays absorption (1), and gastric irritation and bleeding which may result from the erosive action of the crystals of the drug (2), or possibly from its acidity (3), as well as a neurohumoral action (4). It has been shown by Alvarez and Summerskill (5) that there is a causal relationship between salicylate consumption and massive gastrointestinal hemorrhage associated with peptic ulcer. For these reasons, there are a number of proprietary preparations of acetylsalicylate available which are buffered so as to minimize these problems. Several investigators have noted that such buffered preparations are more rapidly absorbed (6, 7). There are also scattered reports that soluble preparations of acetylsalicylic acid or salicylic acid are less irritating and less prone to produce gastric hemorrhage (8, 9). The present investigations were designed to compare the effects of such buffered preparations upon free and total gastric acidity and gastric pH, as compared to unbuffered aspirin.

Received October 7, 1961, from the Departments of Pharmacology and Biochemistry, The George Washington University, School of Medicine, Washington 5, D. C.

Accepted for publication November 3, 1961.

This study was aided by grants from the Miles Laboratories, Inc., Elkhart, Ind.

† Deceased October 1960.

PROCEDURE

For the initial experiments, subjects were selected at random from available laboratory personnel and included six male and one female subject. They were fasted for 12 hours prior to the experiment and then given a test meal consisting of 50 ml. of cold 45% alcohol, to stimulate gastric secretion. Fifteen minutes after the test meal each subject swallowed a stomach tube, and 5 minutes later withdrew a zero-hour sample of 10 ml. of fluid. Immediately after removal of this control sample the drugs were administered. Over a period of 2 weeks each subject received each drug and performed one experiment where only water was administered (control). Effervescent aspirin[1] was administered dissolved in water, two tablets in 250 ml. of water; buffered aspirin[2] and aspirin[3] were taken whole, two tablets followed by 250 ml. of water. All doses were equivalent to 650 mg. of acetylsalicylic acid. Controls received 250 ml. of water only.

Ten-milliliter samples of gastric juice were removed 10, 20, 30, and 40 minutes after administration of the drugs. All samples were tested for pH using pHydrion paper; for free acid by titration with 0.01 *N* sodium hydroxide to a change in color with Töpfers reagent (pH 3.5); and for total acidity by titration to the end point of phenolphthalein (pH 8.4). Free and total acidity are reported in clinical units (milliliters of 0.1 *N* sodium hydroxide required to neutralize 100 ml. of gastric juice).

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In view of the wide variability observed in these subjects' results, the experiments using aspirin and effervescent aspirin were repeated under more carefully controlled conditions and with more accurate procedures for measurement of pH and free and total acidity.

Some of the subjects in the first series of experiments had experienced a great deal of difficulty in swallowing the stomach tube. In order to eliminate the possibility of such trauma affecting the response to the test meal, 35 prospective subjects were tested as to their ability to swallow the stomach tube without difficulty. Twelve were selected as having little or no difficulty and were used in this series of experiments; these included ten male and two female subjects. The test meal was changed to one designed to give better stimulation and consisted of 50 ml. of 14% ethanol to which was added one-half teaspoonful of dry Cream of Wheat to provide mechanical stimulation. The general procedure used was the same except that the drugs were administered with only 150 ml. of water. The pH was measured using the Beckman Zeromatic pH meter. Free and total acidity were measured by titration with 0.01 *N* sodium hydroxide to pH of 3.5 and 8.4, respectively, as measured by the pH meter.

RESULTS

The subjects exhibited a widely varying and generally poor response to the test meal used. In spite of this, marked differences were obtained with the drugs tested (Table I). Effervescent aspirin caused an immediate elevation in gastric pH which reached a maximum of 2.2 pH units above the water control at 20 minutes, and decreased thereafter. A statistical analysis revealed that the gastric pH with the effervescent preparation was significantly higher at 10 and 20 minutes ($P < 0.05$ and < 0.001, respectively) than with the other three preparations. After 20 minutes no differences were seen. The other two salicylate preparations do not affect the pH significantly at any time, nor do they alter free acidity, as compared to the water control. The free acidity drops sharply after the administration of the effervescent drug, the difference being highly significant ($P < 0.01$) at 10, 20, and 30 minutes, and probably significant at 40 minutes ($P = 0.05$). Total acid levels were also compared by the analysis of variance technique, and no differences were found among the drugs.

The second series of experiments, employing a test meal of greater stimulatory capacity and selected subjects, was much more uniform. Effervescent aspirin again produced a marked elevation in pH as compared to aspirin (Table II, Fig. 1) and a marked drop in free acidity (Table II, Fig. 2). An analysis of variance was again performed, followed by comparisons at the individual times by the technique of Scheffe (10). The pH differences were highly significant through 30 minutes ($P < 0.001$) and probably significant at 40 minutes. Differences in free acidity were also significant at 10, 20, and 30 minutes ($P < 0.01$). Total acidities differed only at the 40-minute measurement at a low level of significance ($P < 0.05$).

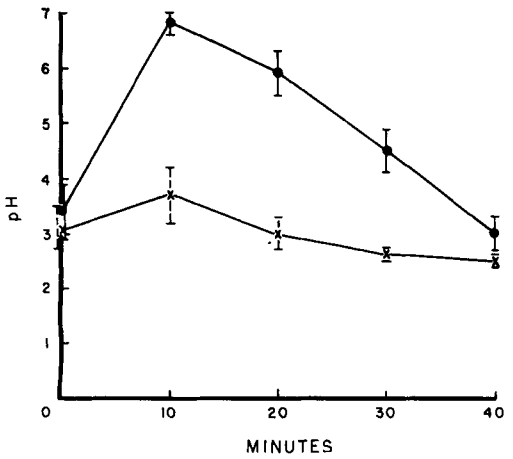


Fig. 1.—Comparison of the effects of 650 mg. of aspirin or effervescent aspirin upon the gastric pH of 12 normal subjects after a standard test meal. Vertical bars represent the standard error of the mean. ●, Effervescent aspirin; ×, aspirin.

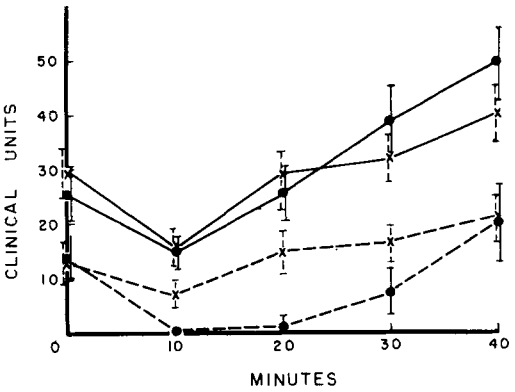


Fig. 2.—Comparison of the effects of 650 mg. of aspirin or effervescent aspirin upon the free and total gastric acidities of 12 normal subjects after a standard test meal. Vertical bars represent the standard error of the mean. ●, Effervescent aspirin; ×, aspirin; — total acidity; - -, free acidity.

DISCUSSION

The results as presented in the tables and figures indicate clearly that effervescent aspirin decreases the free gastric acidity and increases the pH for at least 30 minutes. In contrast, acetylsalicylic acid tablets and buffered aspirin increase the pH only slightly, and depress the acidity to a small degree. This latter effect is presumably due only to dilution of the gastric contents with the ingested water, since these effects did not differ from the controls. Rubin, Pelikan, and Kensler (11) measured intragastric pH levels after the administration of placebos, acetylsalicylic acid, and buffered aspirin, and noted an increase of 0.24 to 0.29 pH units in all cases, these differences not being statistically significant. It will be noted that the pH of those subjects ingesting acetylsalicylic acid tablets or buffered tablets never exceeded 3.7, falling to 2.6 by 40 minutes. This might be expected since at this dosage the buffered aspirin had a neutralization equivalent of only 5 meq. of hydrochloric acid (50 ml. of 0.1 *N* hydrochloric acid). Since the pKa of acetylsalicylate is

TABLE I.—EFFECT OF SEVERAL ASPIRIN PREPARATIONS UPON GASTRIC ACIDITY

Subject	Free Acid Clinical Units 0	10	20	30	40	Total Acid Clinical Units 0	10	20	30	40	pH 0	10	20	30	40
No Drug Administered															
A	23.0	12.0	48.5	63.5	53.5	32.5	15.5	52.5	71.5	53.5	2.1	2.4	1.6	1.4	1.4
B	10.0	22.5	35.5	34.0	39.5	21.5	32.5	50.0	45.0	53.5	2.8	1.7	1.4	1.4	1.4
C	0.0	2.5	24.0	52.5	0.0	6.0	5.5	36.0	61.5	9.5	5.5	4.0	1.4	1.5	4.0
D	0.0	0.0	0.0	0.0	0.0	3.0	1.0	4.0	4.0	8.0	5.5	5.5	5.5	5.0	4.8
E	5.0	5.0	6.0	35.0	34.0	12.0	10.0	3.0	43.0	41.0	3.5	3.0	1.7	1.4	1.7
F	0.0	2.5	8.0	24.0	42.5	9.5	12.5	16.5	36.0	50.0	5.5	4.0	2.4	2.0	1.5
G	0.0	1.0	4.5	7.5	14.5	7.0	4.5	8.0	13.0	18.5	4.0	3.0	3.0	2.5	2.0
Mean	5.45	6.50	18.05	30.80	26.15	13.10	11.65	24.30	39.15	33.45	4.13	3.37	2.43	2.17	2.40
S.E.	3.27	3.06	6.94	8.60	8.06	3.93	3.96	8.15	9.16	7.84	0.53	0.47	0.56	0.50	0.53
Aspirin Tablets															
A	22.0	13.5	37.5	65.0	63.0	33.0	17.0	43.5	76.0	70.0	1.7	1.7	1.4	1.4	1.4
B	0.0	4.5	18.0	27.0	29.5	6.0	11.0	33.5	39.5	33.0	5.0	3.7	2.4	1.8	1.4
C	5.0	2.5	3.5	2.0	8.5	18.5	9.0	15.0	28.0	34.0	2.8	3.0	3.6	3.0	2.8
D	8.0	4.0	2.5	0.0	0.0	23.0	5.5	13.5	5.0	4.0	2.0	2.8	3.8	5.0	5.5
E	0.0	0.0	4.0	8.0	49.0	3.0	5.0	7.5	18.0	59.5	7.5	5.0	3.8	2.8	1.5
F	0.0	4.0	33.5	61.0	72.0	3.5	16.0	40.5	75.0	81.0	5.5	3.0	1.7	1.4	1.4
G	0.0	5.0	9.0	28.5	40.5	11.0	13.0	14.5	36.0	45.5	3.8	3.5	2.5	1.8	1.5
Mean	5.0	4.8	15.50	27.35	32.35	19.0	10.95	24.00	39.65	46.70	4.04	3.24	2.74	2.46	2.21
S.E.	3.07	1.58	5.59	10.15	10.10	4.27	1.79	5.55	10.20	9.82	0.79	0.38	0.38	0.49	0.58
Buffered Aspirin Tablets															
A	10.0	6.0	23.0	34.5	44.0	17.5	1.2	34.0	41.0	56.0	2.0	2.4	1.8	1.4	1.4
B	0.0	0.0	23.0	36.5	17.0	4.5	5.5	38.5	51.5	39.5	7.5	6.0	1.8	1.8	2.4
C	4.5	0.0	7.0	23.0	33.5	8.0	5.5	12.5	36.0	41.0	3.0	4.0	3.0	1.7	1.4
D	8.0	4.5	18.0	30.0	38.0	30.5	10.0	25.0	48.0	61.0	2.1	2.8	1.7	1.4	1.4
E	13.0	10.0	11.5	11.5	6.0	23.0	16.5	16.7	17.0	12.5	2.4	2.4	2.4	2.1	3.0
F	0.0	9.5	23.5	43.5	49.5	1.5	16.0	31.0	43.5	61.0	5.5	2.8	1.7	1.4	1.4
G	0.0	2.0	0.0	12.0	28.0	5.5	8.0	8.0	22.0	41.5	4.0	3.5	4.0	3.0	2.5
Mean	5.05	4.55	15.15	27.30	30.55	14.95	10.50	23.55	37.0	44.65	3.78	3.41	2.34	1.83	1.93
S.E.	2.03	1.57	3.49	4.65	5.76	4.12	1.72	4.40	4.92	6.45	0.78	0.48	0.33	0.22	0.26
Effervescent Aspirin Tablets															
A	14.5	0.0	0.0	0.0	21.0	20.0	8.0	7.0	22.5	36.0	2.1	5.5	5.5	3.8	2.0
B	0.0	0.0	0.0	0.0	0.0	5.0	5.0	9.5	23.5	32.0	5.5	5.5	5.5	4.5	3.5
C	0.0	0.0	0.0	40.5	23.0	5.0	4.5	2.0	55.0	38.5	5.0	5.5	5.5	1.4	1.4
D	0.0	0.0	0.0	0.0	0.0	4.0	6.0	10.5	9.5	13.5	5.5	5.5	5.5	5.0	5.0
E	0.0	3.5	17.5	43.5	44.5	16.0	15.0	37.0	55.0	65.0	3.0	3.0	2.5	1.5	1.5
F	0.0	0.0	0.0	0.0	44.0	6.0	16.0	5.5	13.0	68.5	5.5	4.0	4.0	4.5	1.5
G	21.5	0.0	0.0	0.0	19.0	22.0	4.0	8.5	39.0	81.5	1.7	5.5	5.5	4.0	1.4
Mean	5.15	0.50	2.50	12.00	24.50	11.85	8.35	11.45	31.05	47.85	4.04	4.93	4.86	3.53	2.33
S.E.	3.41	0.50	2.50	7.75	8.47	2.61	1.91	4.40	7.11	9.12	0.65	0.38	0.45	0.50	0.53

TABLE II.—EFFECT OF ASPIRIN AND EFFERVESCENT ASPIRIN ON GASTRIC ACIDITY

Subject	Free Acid, Clinical Units					Total Acid, Clinical Units					pH				
	0	10	20	30	40	0	10	20	30	40	0	10	20	30	40
Aspirin Tablets															
A	10.0	0.0	18.9	20.0	17.3	20.5	7.3	35.5	42.3	32.8	2.6	3.8	2.5	2.6	2.3
B	19.6	8.2	16.6	27.3	32.8	38.2	16.4	37.3	45.5	66.3	2.3	2.8	2.5	2.2	2.5
C	3.6	7.5	2.8	14.1	12.7	15.9	15.9	16.6	25.0	30.0	2.8	2.8	3.2	2.5	2.5
D	25.5	10.9	15.9	8.2	18.7	44.6	20.5	34.8	23.7	37.8	2.3	2.5	2.5	2.8	2.3
E	0.0	0.0	0.0	0.0	10.0	12.7	6.4	19.6	10.5	26.4	4.0	3.6	3.5	3.4	2.6
F	34.1	16.4	32.3	23.2	53.0	52.8	30.0	50.0	36.4	74.2	2.8	2.4	2.2	2.2	2.0
G	8.2	3.2	8.6	12.7	10.5	30.9	9.1	13.2	25.0	29.6	2.7	2.9	2.7	2.5	2.6
H	7.7	0.0	2.7	10.0	12.7	25.9	2.3	15.9	27.8	34.9	2.8	7.1	3.1	2.6	2.7
K	3.6	2.3	19.6	3.6	10.5	24.6	13.4	30.5	16.8	24.6	3.0	3.2	2.3	2.9	2.6
L	6.4	8.2	10.9	16.8	18.7	29.6	20.7	26.4	35.0	34.6	2.9	2.8	2.7	2.6	2.5
M	0.0	0.0	0.0	5.9	4.6	2.3	2.0	4.8	22.3	23.2	7.2	7.9	6.4	2.7	2.8
O	40.9	29.2	45.5	46.4	44.6	55.0	41.9	60.7	71.4	69.6	2.1	2.2	2.0	2.1	2.2
Mean	13.3	7.2	14.5	15.7	20.5	29.4	15.5	28.8	31.8	40.3	3.12	3.65	2.97	2.59	2.48
S.E.	3.94	2.48	3.96	3.64	4.34	4.63	3.39	4.64	4.63	5.34	0.40	0.54	0.34	0.10	0.07
Effervescent Aspirin															
A	10.0	0.0	0.0	0.0	0.0	20.0	21.4	29.1	43.2	45.5	2.5	7.2	5.3	4.1	4.6
B	16.4	0.0	0.0	0.0	30.5	25.5	5.5	10.9	51.0	68.7	2.4	7.2	6.8	3.9	2.4
C	11.4	0.0	0.0	0.0	10.9	21.4	6.8	9.1	32.1	46.4	2.6	7.3	6.7	5.2	2.9
D	32.3	0.0	0.0	19.6	59.6	51.0	24.6	28.7	66.0	77.4	2.2	6.4	6.1	3.0	2.2
E	15.0	0.0	0.0	0.0	0.0	25.5	23.7	34.6	34.1	29.6	2.4	6.2	6.5	5.5	4.8
F	53.7	0.0	17.3	50.0	74.9	64.6	35.5	70.5	89.2	99.6	2.2	5.5	2.6	2.0	1.8
G	1.4	0.0	0.0	0.0	13.5	19.1	9.6	21.8	36.4	41.9	3.3	7.1	6.4	5.3	2.8
H	0.0	0.0	0.0	0.0	15.0	6.4	12.7	19.6	28.2	32.8	7.1	6.7	6.0	4.9	2.5
K	11.4	0.0	0.0	15.9	0.9	24.6	5.5	43.7	27.0	16.8	2.7	6.7	4.3	2.5	3.2
L	0.0	0.0	0.0	0.0	12.7	16.4	6.8	16.4	29.6	54.6	3.5	7.1	6.8	4.8	2.5
M	0.0	0.0	0.0	0.0	0.0	4.6	8.6	7.7	13.5	21.8	7.0	7.6	7.3	6.9	4.3
O	10.5	0.0	0.0	0.0	19.1	27.3	16.4	15.9	15.0	56.0	2.8	6.6	6.5	6.3	2.4
Mean	13.5	0.0	1.44	7.1	19.8	25.5	14.8	25.7	38.8	49.3	3.39	6.8	5.94	4.53	3.03
S.E.	4.89	0.0	1.44	4.37	7.00	4.09	2.78	5.15	6.2	6.92	0.51	0.16	0.38	0.43	0.29

3.49, this means that a large proportion of the drug is unionized and of relatively low solubility throughout the period of measurement. The solubility of the drug is about 6.6 Gm./L. at 37° at pH 3 in dilute hydrochloric acid, so that all of the 650 mg. could be in solution in the volume of water used. However, the local pH at the mucosal wall and the diffusion constants of solid acetylsalicylic acid as it goes into solution would modify the rate of solution. Edwards (1) made theoretical calculations concerning such factors, and Nelson and Schaldemose (12) confirmed these experimentally by showing that acetylsalicylic acid in aqueous solution was much more rapidly absorbed than a comparable amount in tablet form. In contrast, after the administration of effervescent aspirin, the pH remained above 4.5 up to 30 minutes so that the drug would be more than 90% in the ionized form throughout this period. The effervescent preparation contains about 10 times more buffer, equivalent to 48 meq. It will also be noted that this large amount is not reflected in the total acid titrations; in the second experiment at 40 minutes, the increase was only 0.9 meq./100 ml. of gastric juice. This must result from the relatively rapid passage of a large proportion of the buffered gastric juice into the intestine. Moreover, Bolton (13) has demonstrated that citrate enhances solubility of acetylsalicylic acid, so that in pH 4.5 sodium citrate, acetylsalicylate is soluble to the extent of 32.4 Gm./L. at 30°. Obviously, solubility is not a problem with this buffered preparation and probably accounts for its more rapid absorption, reaching a peak at 30 to 45 minutes (6, 7). The enhanced solubility may very well reduce the gastric irritation associated with plain aspirin. In a group of 103 patients, Alvarez and Summerskill (5) incriminated salicylates in over 40% of cases admitted for hematemesis and melena connected with peptic ulcer. Stubbe (8), in studying patients with rheumatoid arthritis, observed that about 70% of these patients and a control population gave positive benzidine tests for occult blood in the stool regardless of the amounts of aspirin ingested. Enteric coated aspirin gave positive results also, but when positive patients (on aspirin) were changed to sodium salicylate, the benzidine tests became negative. In recent work, Stubbe (14, unpublished) has shown that positive benzidine tests for occult blood occurring when patients were on aspirin became negative when the medication was changed to effervescent aspirin. Pierson, *et al.* (9), employing chromium-tagged red cells, made similar observations with calcium aspirin complex and choline salicylate. Muir and Cossar (2) have seen crystals of acetylsalicylate for as long as several hours after tablet administration. Davison and Guy (unpublished) observed more occult blood produced in pyloric pouch dogs at acid pH values after the administration of acetylsalicylate as opposed to neutral pH. It would appear, therefore, that the erosive properties of the crystalline form of acetylsalicylic acid may account for at least a part of its hemorrhagic properties.

Hurley (15, unpublished), using gastroscopic observations in dogs and substantiated by photographs, has evaluated the local effects of aspirin and soluble effervescent aspirin on the gastric mucosa over a 2-week period. Aspirin produced initial characteristic erosions of the mucosa with hemorrhage, which cleared after 5 days and remained so for the balance of the dosage period. Administration of soluble effervescent aspirin over the same period had no effect on the mucosa. If aspirin is administered following a previous period of effervescent aspirin, the characteristic erosion and hemorrhage occur which clears up after 1 week.

Winkelman (16) suggests that while aspirin and associated salicylates are incriminated in bleeding, with or without peptic ulcer, no proved effect has been demonstrated, but proposed that factors responsible for ulcer production, such as impaired mucosal resistance or increased acid-pepsin secretion, may enhance the development of acute ulceration from salicylate-induced gastritis in some patients.

As Brodie and Hogben (17) have pointed out, it is apparently the unionized form of the drug which is absorbed, and Davison and Guy (unpublished) have confirmed this in dogs with pyloric pouches. Consequently, the buffered forms of acetylsalicylic acid should be absorbed more slowly on the ionic basis but, apparently, the enhanced solubility and consequent distribution over the entire stomach wall, as well as the more rapid penetration into the intestine, overcome this deficiency. Acetylsalicylic acid can even be absorbed within the intestine despite apparently unfavorable pH since, according to Brodie and Hogben (17), the pH of the intestinal wall can be as low as 5.3.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product II

Effect of Sampling and Bulk Mix Heterogeneity on Tablet Variation

By EDWARD R. GARRETT† and EDWARD C. OLSON‡

The coulometric bromine oxidation procedure for *o*-methoxy-α-methylphenethylhydrazine has been applied to the analysis of two experimental tablet preparations and one lot of bulk drug. The results show that the variation from tablet to tablet is significantly greater than the error in the assay, that random sampling of bulk drug should be rigidly controlled, that the micro-heterogeneity of a tablet mix may exceed the variability among tablets. The present investigation points out the need for statistically designed studies to determine the uniformity of the drug content.

One of the major production and control interests in the pharmaceutical industry is the preparation of reproducible dosage forms. The variables encountered are the degree of homogeneity of the bulk drug and the pharmaceutical mixture of the drug and excipient, the reproducibility of the dose per unit dosage form, and the relation of these factors to the error in the assay of bulk drug, formulation mix, and final dosage form.

This paper considers the detailed design and statistical evaluation of data obtained on an exploratory batch of tablets to delineate completely the above stated problems.

A coulometric titration procedure with electrolytically generated bromine had been devised to assay the pharmacologically active drug, *o*-methoxy-α-methylphenethylhydrazine in acetic acid-water-methanol and is reported in the literature (1).

The statistical methods are as given in standard texts (2) and the prior paper in this series (3).

EXPERIMENTAL

The coulometric titration with electrolytically generated bromine of *o*-methoxy-α-methylphenethylhydrazine, I, has been published by Olson (1) and the procedures used in these studies were identical with those described. For single tablet assay, the tablet was thoroughly ground, and weighed portions of this mix were analyzed. Analysis of I without excipients and with the excipients of tablet preparations A and B shows no interference with the assay procedure (Table I). The consistent positive error in these assays has been shown to be due to slight hydrazine contamination.

The details of the statistical designs for the studies

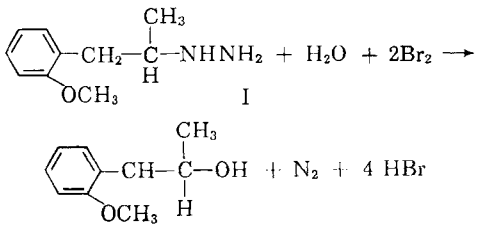


TABLE I.—EFFECT OF EXCIPIENT ON ASSAY OF DRUG

Excipient/12 mg. of Drug	% Drug[a]
None	105.14
	105.09
Preparation A	
8 mg. citric acid	
14.5 mg. starch	106.21
210 mg. terra alba	105.18
3.0 mg. calcium stearate	
Preparation B	
8.0 mg. citric acid	
19.5 mg. starch	
180 mg. mannitol	105.04
4.0 mg. calcium stearate	106.18
5.0 mg. talc	

[a] Hydrazine contaminant gives positive error (1).

TABLE II.—PER CENT *o*-METHOXY-α-METHYLPHENETHYLHYDRAZINE IN 25 SAMPLES OF LOT A[a]

Day	Sample 1	2	3	4	5
1	101.85	100.82	102.54	101.21	102.75
	104.46	101.22	101.75	101.91	102.19
2	102.87	101.50	102.85	102.35	102.56
	102.09	102.22	102.53	102.28	102.41
3	103.36	100.30	100.18	107.67	97.84
	101.43	101.00	97.21	99.77	99.79
4	100.28	100.66	98.95	100.25	97.88
	103.78	101.23	101.79	100.88	101.46
5	100.58	101.75	105.58	103.15	103.69
	102.32	103.44	103.31	102.48	103.53
			Average	101.518	

[a] The request was for random samples of the lot. The duplicates are from a blended, homogenized sample. Five samples were run on each of five successive days.

Received September 5, 1961, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

Accepted for publication October 18, 1961.

The technical assistance of Mr. R. S. Shaw, and Mr. E. Markovich in the operation of the E102 Burroughs Digital Computer is gratefully acknowledged.

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TABLE III.—ANALYSIS OF VARIANCE OF TABLE II DATA[a]

Source of Variation	DF	SS	MS	Components of Variance	F Test
Total	49	166.96			
Among days	4	38.61	9.651	$\sigma^2 + 2\sigma_s^2 + 10\sigma_D^2$	$F = 3.15$ (5% F is 2.9); significant.
Within days	20	61.23	3.061	$\sigma^2 + 2\sigma_s^2$	Not significant
Between duplicates	25	67.12	2.684	σ^2	

Estimated variances

$\sigma^2 = 2.684$; $\sigma = 1.64$

$\sigma_s^2 = 0.189$; $\sigma_s = 0.43$

$\sigma_D^2 = 0.659$; $\sigma_D = 0.81$

$\sigma_t^2 = \sigma^2 + \sigma_s^2 + \sigma_D^2 = 3.532$; $\sigma_t = 1.88$

[a] Duplicate assays among and within days.

on the homogeneity of the bulk drug and tablets with the evaluation of assay errors are given in the next section.

RESULTS AND DISCUSSION

Evaluation of Variability among Assays and Days of Assay of the Bulk Drug.—The coulometric assay of *o*-methoxy-α-methylphenethylhydrazine in 25 samples of the bulk drug, lot A, are given in Table II. The high results (>100%) have been shown to be due to hydrazine contaminants (1). The request was made that this lot of bulk drug be randomly sampled. Each of the 25 samples was homogenized and micronized, and two assays were run on each sample. As received, 5 samples were run in duplicate on each of five days.

The Table III analysis of variance of the data of Table II demonstrated that the variation among the samples run on the same day is not significantly greater than the variations between duplicates. However, the variation among days is significantly greater than the variation among the daily samples. This implies a daily variation in the assay or a bias in the samples assigned to be assayed on a given day.

TABLE IV.—ASSAY DATA OF DRUG IN TABLETS OF PREPARATION A

Tablet No.	Tablet Wt., mg.	Replicate Assays mg. Drug/100 mg. of Tablets	
1	249.6	3.626	3.699
2	250.9	3.882	3.944
3	251.1	4.396	4.227
4	249.5	4.100	4.026
5	249.7	4.189	4.119
6	250.9	4.322	4.261
7	248.4	4.078	4.019
8	244.3	3.982	3.967
9	247.6	3.942	3.845
10	244.9	3.639	3.682
Average	248.7	4.000	

$\sigma^2 = 5.79$, $\sigma = 2.41$, % of mean = 0.969 for tablet wt.

Random Samples of Blend	
1	4.334
2	4.057
3	3.274
4	3.296
5	3.635
Average	3.719
σ^2	0.2191
σ	0.468
S. D.: % of Mean	12.6

TABLE V.—ASSAY DATA OF DRUG IN TABLETS OF PREPARATION B

Tablet No.	Tablet wt., mg.	Replicate Assays mg. Drug/100 mg. of Tablets	
1	230.2	4.042	3.826
2	232.3	4.406	4.372
3	228.0	4.358	4.382
4	231.5	4.322	4.284
5	226.1	4.185	4.095
6	229.0	4.012	3.881
7	230.0	4.178	4.134
8	228.1	4.167	4.173
9	236.1	4.469	4.694
Average	230.1	4.221	

$\sigma^2 = 8.553$, $\sigma = 2.93$, % of mean = 1.27 for tablet wt.

Random Samples of Blend	
1	4.573
2	4.937
3	3.900
4	4.825
5	4.818
Average	4.611
σ^2	0.1755
σ	0.419
S. D.: % of Mean	9.1

This latter could have been the case since no specific instructions as to how to sample randomly were given. For example, samples run on the first day may have been from the top fraction of the bulk drug, on the second day may have been from a second layer, etc. Future designs should be fully cognizant of this source of possible bias.

The estimated error in (standard deviation) per cent of the mean among samples within days is 1.64%. The variation ascribed among days is 0.81%. The standard deviation of any one sample (pooled among days and assays) may be estimated as 1.88%.

Evaluation of Variability among Tablets from Two Different Preparations, A and B.—Two tablet preparations were studied. The data are given in Tables IV and V and consist of the weight of 10 individual tablets and two replicate assays of these tablets. The tablets were halved and the halves randomized so that the operator had no knowledge of whether he was assaying the same or another tablet.

In addition, a sample of the bulk mix obtained prior to the tableting was strewn along the table top and at equal linear intervals, five samples were obtained and assayed.

TABLE VI.—ANALYSIS OF VARIANCE OF TABLE IV, PREPARATION A

Source of Variation	SS	DF	MS	Components of Variance	*F* Test
Among tablets	0.91408825	9	0.1015654	$2\sigma_1^2 + \sigma^2$	$F = 27.9$, significant at 0.001 level
Replicates	0.03774750	10	0.00377475	σ^2	
Total	0.95183575	19			

Estimated variances

$\sigma^2 = 0.0037748 \therefore \sigma = 0.0615$ S. D., % of mean = 1.54

$\sigma_1^2 = 0.0489 \therefore \sigma_1$ 0.221 S. D., % of mean = 5.52

$\sigma_t^2 = \sigma^2 + \sigma_1^2 = 0.0527 \therefore \sigma_1 = 0.230$ S. D., % of mean = 5.60

TABLE VII.—ANALYSIS OF VARIANCE OF TABLE V, PREPARATION B

Source of Variation	SS	DF	MS	Components of variance	*F* Test
Among tablets	0.716431	8	0.089533	$2\sigma_1^2 + \sigma^2$	$F = 12.6$ significant at <0.001 level
Replicates	0.063845	9	0.0070944	σ^2	
Total	0.780276	17			

Estimated variance

$\sigma^2 = 0.0070944 \therefore \sigma = 0.0843$, S. D., % of mean = 2.00

$\sigma_1^2 = 0.04123 \therefore \sigma_1 = 0.203$, S. D., % of mean = 4.80

$\sigma_t^2 = \sigma^2 + \sigma_1^2 = 0.04832 \therefore \sigma_t = 0.220$, S. D., % of mean = 5.21

The analyses of variance for the preparations are given in Tables VI and VII. The variation among tablets for the two preparations, A and B, was similar. The variations in drug assay (as standard deviation, per cent of mean) from tablet to tablet were ±5.5 and 4.8%, respectively. The respective estimates of assay error were 1.54 and 2.00%. These latter values agree well with the 1.64% among assays within days and the 1.88% among assay and days estimated from the previous study on the bulk drug.

The variations in tablet weight are small, ±1 and ±1.3%, respectively, for the two preparations.

It should be pointed out that the tablet-to-tablet variation of drug content is actually larger than that given in Tables IV and V, since the drug assay is given in terms of mg./100 mg. of tablet.

The blend prior to tableting shows an even greater heterogeneity than from tablet to tablet, 12.6 and 9.1%, respectively. This is significantly greater (by *t* test) than the variation among tablets. A valid explanation is that tableting tends to average out some of the inherent microscopic heterogeneity of the pretableting blend.

CONCLUSIONS

The variations in assay of *o*-methoxy-α-methylphenethylhydrazine from tablet to tablet far exceed the error in the assay for the preparations studied. This is highly significant. The drug potency varies 5% from tablet to tablet when considered on a per-unit weight basis Variation between the preparations shows no effect of the excipients. The assay error is only 1.5–2.0%.

The microscopic heterogeneity of the pretableting blend is significantly very much greater than that in the tablets and runs to ±9.1–12.6% in per cent standard deviation. (This includes assay error.)

Assay studies on the bulk drug substantiated the above assay error, 1.64% (per cent standard deviation). Significant variation in assay with days of assay may be attributed to possible bias in the sampling of drug from the bulk lot on the various days. Properly, this portion of the experiment should be repeated to substantiate or negate this possible source of variability.

Studies such as these serve an important function in that they may guide the proper future preparation of tablets to be used in clinical drug evaluation or to be placed on the market.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product III

Statistical and Economic Evaluation of the Three-Component Infrared Spectrophotometric Assay of Aspirin Anhydride

By EDWARD R. GARRETT† and JAMES L. JOHNSON

An experimental design of a series of three-component infrared spectrophotometric assays of various preparations of aspirin anhydride is statistically analyzed. Methods are shown for the proper choice of standards and the economical design of assays for a desired statistical confidence. Information as to stability and significance of physicochemical properties, such as melting point, is also obtained.

In previous papers of this series (1, 2), the statistical evaluation of final and bulk drug assays by ultraviolet spectrophotometry (1) and coulometry (2) was considered. The studies on aspirin anhydride (AA) as a possibly superior form for the oral administration of aspirin (3–6) necessitated the preparation of various batches. A three-component infrared spectrophotometric procedure was devised (4) to assay for the aspirin anhydride, aspirin, and salicylic acid in such material.

In general, three-component infrared spectrophotometric assays against synthetic standards are not as precise as the previous types of instrumental analysis discussed. Questions that should be answered concern the precision of such assays, the choice of the proper standard, the economics of replication within and among days of assays.

An experimental design to permit such conclusions is considered in this paper. The validity and reliability of the melting point as an indicator of purity is also considered. The weight loss and initial purity of the preparations are considered in light of the tendencies of the preparations toward instability. The statistical methods used are as discussed in standard texts (7).

EXPERIMENTAL

Four separate lots, A, B, C, and D, of aspirin anhydride which had been stored since preparation were used. They were sampled from the center of 100-Gm. amounts which had been sealed in brown glass bottles. The age of these lots and the analytical data obtained at the time they were originally prepared are given in Table I.

An additional lot, E, in use in Lab A as the assay standard, was included in the study. Ten samples, two of each lot, were submitted to Lab A on each of 5 days with requests for aspirin, salicylic acid, and aspirin anhydride assays of each. The coding of the samples was such (see Table II) that the operator was unaware of the duplication or that the same sample was submitted on sequential days. On each of the days of assay, aspirin anhydride, lot E, aspirin, and salicylic acid were run to serve as standards for the three-component assays on that day. The method of infrared assay was as given in the literature (4). The results of these assays are given in Table II.

Simultaneously with these studies, the lots were submitted on various days for weight loss on drying and melting point. These data are given in Tables III and IV.

RESULTS AND DISCUSSION

The analyses of variance of the anhydride, aspirin, and salicylic acid assays in AA are given in Tables V, VI, and VII. With all three, the assay variation among days exceeds the error among duplicate assays on the same day. Thus, assay duplication on any given day against the same standards may not be warranted if any obtained assay value is to be considered as characteristic of a particular preparation when assayed on any day.

The estimated standard deviations of a single infrared assay, including the error, day by lot interaction, and variation among days are ±0.9% for aspirin anhydride, ±0.4% for aspirin, and ±0.2% for salicylic acid for 87–100% pure anhydride. These standard deviations are given in per cent of the total sample, not in per cent of the component. The 95% confidence limits may be estimated by doubling these values.

Inspection of the data in Table II in comparison with Table I clearly shows that lot A would serve as a better standard than lot E.

In addition, appended to Tables V, VI, and VII are estimates of the components of variance for the data calculated from the standards run on the same day and for the average of the standards over the 5 days. In the cases of salicylic acid and aspirin assays of the AA, it apparently makes no difference whether a separate daily set of standards or the averages are used. However, the variability among days of assays for AA assay vanishes when the average of the standards is used and the total error among assays lessens. Thus, it is recommended that the same absorptivity constants be used in the calculation of all assays at all times. A set of

Received September 5, 1961, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication October 18, 1961.
The operation of the E102 Burroughs digital computer by Mr. E. Markovich is gratefully acknowledged.
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TABLE I.—DATA ON ASPIRIN ANHYDRIDE (AA) LOTS AT TIME OF PREPARATION

Lot No.	A		B		C		D	
Age, mo.	12.0		9.2		8.0		7.5	
M.p., ° C.	85–86.5		77–81		81–83		82–84.5	
Lab	A	B	A	B	A	B	A	B
AA, %	101.0	100.3	96.0	96.3	97.5	99.8	99.1	98.7
Aspirin, %	0.2	0.1	3.0	1.8	2.1	1.0	1.0	0.8
Salicylic acid, %	0.1	0.1	0.3	0.6	0.0	0.2	0.4	0.1

TABLE II.—INFRARED ASSAYS OF ASPIRIN ANHYDRIDE (AA), ASPIRIN, AND SALICYLIC ACID IN 5 LOTS OF AA REPLICATED TWICE DAILY FOR 5 DAYS[a]

Day Submitted	Lot A Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot B Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot C Code No.	AA, %	Asp., %	Sal., %	Total, %
1	X110	99.3	0.7	0.0	100.0	X123	95.0	3.5	0.3	98.8	X131	87.0	9.0	0.4	96.4
	X110	99.0	0.7	0.0	99.7	X120	94.6	3.5	0.0	98.1	X130	87.3	9.1	0.1	96.5
2	X259	99.9	0.4	0.0	100.3	X215	96.4	2.8	0.0	99.2	X221	88.0	8.7	0.2	96.9
	X250	100.0	0.6	0.0	100.6	X210	97.2	2.4	0.0	99.6	X220	87.6	9.0	0.0	96.6
3	X353	100.8	0.3	0.5	101.6	X338	95.7	2.5	0.5	98.7	X317	87.7	9.1	0.6	97.4
	X360	100.1	0.1	0.5	100.7	X370	94.7	2.8	0.3	97.8	X340	87.7	8.9	0.6	97.2
4	X442	99.6	0.5	0.2	100.3	X453	94.4	2.8	0.0	97.2	X435	88.1	8.9	0.7	97.7
	X430	99.9	0.3	0.2	100.4	X460	94.4	2.8	0.0	97.2	X420	88.5	8.7	0.6	97.8
5	X537	99.5	0.3	0.0	99.8	X553	93.4	2.8	0.1	96.3	X549	86.2	9.0	0.3	95.5
	X520	98.7	0.4	0.0	99.1	X560	94.5	2.7	0.2	97.4	X580	86.4	9.5	0.3	96.2
Av.	...	99.7	0.43	0.14	100.3	...	95.0	2.86	0.14	98.0	...	87.5	9.0	0.38	96.8

Day Submitted	Lot D Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot E Code No.	AA, %	Asp., %	Sal., %	Total, %
1	X145	96.7	2.8	0.1	99.6	X157	98.1	1.9	0.2	100.2
	X140	96.9	2.2	0.4	99.5	X150	98.1	1.9	0.2	100.2
2	X232	98.2	1.3	0.0	99.5	X241	98.1	1.2	0.0	99.3
	X230	99.1	1.5	0.0	100.6	X240	99.6	0.7	0.0	100.3
3	X326	97.0	1.4	0.4	98.8	X348	99.3	0.8	0.4	100.5
	X380	98.3	1.5	0.4	100.2	X320	99.2	0.8	0.4	100.4
4	X419	97.5	1.3	0.0	98.8	X422	99.6	1.1	0.3	101.0
	X400	98.2	1.2	0.1	99.5	X410	99.6	0.3	0.1	100.0
5	X527	96.6	1.6	0.0	98.2	X512	97.9	0.7	0.2	98.8
	X540	96.3	1.5	0.0	97.8	X530	97.7	0.9	0.1	98.7
Av.	...	97.5	1.63	0.14	99.3	...	98.7	1.03	0.19	99.4

[a] Calculated on the basis of the standards for that particular day.

TABLE III.—WEIGHT LOSS OF ASPIRIN ANHYDRIDE LOTS ON DRYING

Lot	Day Completed[a]	Weight Loss, %
A	8	0.06
	9	0.03
	10	0.04
B	8	0.13
	8	0.22
	10	0.13
C	8	0.24
	10	0.21
	10	0.27
D	9	0.08
	8	0.13
	10	0.20
E	6	0.08
	10	0.02
	10	0.04

[a] The three samples from each lot were respectively submitted on days 3, 5, and 8. The drying was started one day before date of completion.

standards may be run on a given day just to check on being within the control range of the series of standards.

It is interesting to note that lot A has shown no significant degradation for 12 months since its preparation, whereas lots B, C, and D prepared since that time and initially of less purity, have shown some run-down. Lot C has degraded as much as 10%. This confirms the effect of impurities on stability as previously noted (4). Comparison of weight loss values in Table III with the infrared assay (Table II) clearly shows that weight loss, presumably moisture, correlates with instability, as expected from previous studies (4). Melting points correlate with purity as expected. The best preparation, lot A, (Table II) of 99.7% AA has the highest and most reproducible melting point range, 83.4–83.8°, whereas the worst, lot C, of 87.5% AA, has the lowest melting point and the widest range. An individual melting point determination is most valid in the lower limit and least valid in the upper limit (see Table IV).

The estimated component of variance for variation among days of AA assay is $\sigma_D^2 = 0.43$, and for the error among assays on the same day is $\sigma_E^2 = 0.204$. The estimate of variance, σ_{AV}^2 for the average of nk assays could be

$$\sigma_{AV}^2 = \sigma_E^2/nk + \sigma_D^2/k \qquad \text{(Eq. 1)}$$

where n is the number of assays based on one set of standards or run on a given day, and where k is the number of days of assay. The variation among days is assumed to be independent of n. Equation 1 excludes the day × lot variation.

TABLE IV.—MELTING POINTS OF ASPIRIN ANHYDRIDE LOTS

Lot	Day Completed[a]	Melting Point Results, °C.	
A	11	83.4 –83.7	83.5–83.7
		83.5 –83.7	
	11	83.2 –83.7	83.3–83.8
		83.2 –83.8	
		83.4 –83.85	
B	10	79.2 –80.0	79.2–79.9
		79.2 –79.7	
	11	79.25–81.2	79.4–81.5
		79.3 –81.7	
		79.65–81.7	
C	10	78.0 –78.9	78.1–78.9
		78.2 –78.8	
	12	77.3 –83.5	77.6–84.6
		77.8 –83.9	
		77.7 –82.5	
		77.5 –88.4	
D	10	82.0 –82.4	82.1–82.5
		82.2 –82.6	
	12	81.6 –83.1	81.6–82.9
		81.7 –82.7	
		81.4 –83	
E	10	82.0 –82.5	82.1–82.5
		82.0 –82.3	
		82.3 –82.6	
	12	82.2 –96	82.1–96.4
		81.9 –96.8	

[a] The two samples from each lot were respectively submitted on days 3 and 5, each to be run at least in duplicate.

TABLE V.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR ASPIRIN ANHYDRIDE IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S.
Days (D)[c]	19.12	4	4.78	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	966.37	4	241.6	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	8.38	16	0.524	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	5.10	25	0.204	σ_E^2
Total	998.96	49	20.39	

[a] Lots among days, 5; lots, 5; and replicates, 2. [b] F test: days × lots: error = 2.57; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 461; 0.1% F = 7.9 ∴ significant at ≪0.1% level. Days: days × lots = 9.13; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.43$ ($\sigma D^2 = 0$ if av. of daily standard used). [d] Estimated variance; $\sigma DL^2 = 0.16$ ($\sigma DL^2 = 0.15$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.204$ ($\sigma E^2 = 0.212$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.76$ ∴ $\sigma = 0.89\%$ ($\sigma = 0.60\%$ if av. of daily standards used).

Equation 1 may be combined with a cost function to determine the most economic distribution of assays among and within days. A possible cost function could be

$$C = nkc_1 + kc_2 \qquad \text{(Eq. 2)}$$

where C is the total cost, c_1 is the cost of a replicate assay per day, and c_2 is the cost of setting up the assay on any given day. In general c_2 far exceeds c_1, but for $k = 1$, σ_{AV}^2 must always exceed σ_D^2, no matter the number of replicates.

TABLE VI.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR ASPIRIN IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S.
Days (D)[c]	3.80	4	0.950	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	482.62	4	120.65	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	1.747	16	0.1092	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	1.09	25	0.0436	σ_E^2
Total	489.25	49	9.985	

[a] Lots among days, 5; lots, 5; and replicates, 2. [b] F test: days × lots: error = 2.51; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 1.104; 0.1% F = 7.9 ∴ significant at ≪0.1% level. Days: days × lots = 8.70; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.084$ ($\sigma D^2 = 0.075$ if av. of daily standards used). [d] Estimated variance; $\sigma DL^2 = 0.033$ ($\sigma DL^2 = 0.029$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.044$ ($\sigma E^2 = 0.040$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.131$; $\sigma = 0.36\%$ ($\sigma = 0.38\%$ if av. of daily standards used).

TABLE VII.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR SALICYLIC ACID IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S
Days (D)[c]	1.077	4	0.2692	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	0.433	4	0.1082	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	0.3852	16	0.0241	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	0.2150	25	0.0086	σ_E^2
Total	2.1100	49	0.0431	

[a] Lots among days, 5; lots, 5 and replicates, 2. [b] F test: days × lots: error = 2.80; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 4.50; 5% F = 3.0 ∴ significant at 5% level. Days: days × lots = 11.2; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.0245$ ($\sigma D^2 = 0.046$ if av. of daily standards used) [d] Estimated variance; $\sigma DL^2 = 0.0078$ ($\sigma DL^2 = 0.011$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.0086$ ($\sigma E^2 = 0.0078$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.0409$ ∴ $\sigma = 0.204$ ($\sigma = 0.254$ if av. of daily standards used).

As was developed in an analogous situation (8), the condition,

$$n = \sqrt{c_2/c_1}\,(\sigma_E/\sigma_D) \qquad \text{(Eq. 3)}$$

specifies the number of replicates per day for the minimum standard error, σ_{AV}, of the assay at a specified total cost C, where the value of n from Eq. 3 substituted into a rearranged Eq. 2 determines the number of days, k, of n assays.

The condition of Eq. 3 also specifies the value of n for a specifically desired σ_{AV} at minimum cost where this value of n substituted into a rearranged Eq. 1 determines the number of days, k, of n assays.

CONCLUSIONS

The reliability of the infrared assay for aspirin anhydride, salicylic acid, and aspirin is obtained from the studies, and statistical evaluations are

given. The estimated standard deviations of a single infrared assay of aspirin anhydride are AA, ±0.9%; aspirin ±0.4%; and salicylic acid, ±0.2%; in terms of per cent of the total sample. The 95% confidence limits may be estimated by doubling these values. This is based on a new set of standards run on each day. This information demonstrates the foolishness of reporting apparent values by tenths of per cent. For example, if the obtained numerical value of salicylic acid and aspirin in one assay ranges from 0.0 to 0.4%, the amounts present are actually immeasurable and may be considered absent by the sensitivity of the assay.

The greatest assay variation is among days of assay rather than among replicates on a given day. If a routine control procedure is put into effect, assay duplication on the same day is an unnecessary expense. If the most precise assay estimate is desired at minimum cost, special consideration must be given to assaying the sample for several days.

The use of one set of standards or an average value for standards gives less total variability in the AA assay, ±0.6%, and no apparent variation among days. The variability in the aspirin and salicylic acid assays is not significantly changed by the use of either daily or averaged standards.

A comparative study such as this permits the choice of the better standard. For example, lot A should be preferred to lot E. Lot A, the oldest of the lots but of the highest initial purity, has shown no significant degradation for over a year's time. Less initial purity results in greater degradation. It is also shown that low per cent weight loss (considered as low moisture content) correlates with the higher stability.

The correlation of melting point with purity permits the establishment of a desired m.p. and expected error. The lower limit for good AA should be 81.8 ± 0.2°.

The insignificance of salicylic acid in these AA lots of varied purity show that little definition is lost on ignoring it. In fact, a statistical evaluation of data assayed by a two-component assay, for aspirin and AA only, gave less error of estimation.

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Antibacterial Activity of Mixtures of Quaternary Ammonium Compounds and Hexachlorophene

By G. R. WALTER and W. S. GUMP

Hexachlorophene and several quaternary ammonium compounds in admixture were evaluated for antibacterial activity by *in vitro* techniques commonly employed for the evaluation of lotions, creams, and ointments. It was observed that a maximum decrease in activity occurred as the components of the mixture approached equimolar ratios. The formation of a water-insoluble complex tends to diminish the antibacterial activity of mixtures when tested by broth dilution or agar plate techniques.

Numerous examples of the inactivation of bactericidal cationic substances may be found in the literature and several have been reviewed by Lawrence (1).

The anionic nature of hexachlorophene would also suggest a lesser antibacterial action in the presence of quaternary ammonium compounds. The problem of ascertaining the extent of inactivation, if any, was undertaken because of the possible usage of both hexachlorophene and quaternary ammonium compounds in items such as lotions, powders, and creams. The methods selected for bacteriological evaluation were those commonly associated with the *in vitro* evaluation

Received January 4, 1962, from Sindar Corp., Delawanna, N. J.
Accepted for publication January 26, 1962.

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Numerous examples of the inactivation of bactericidal cationic substances may be found in the literature and several have been reviewed by Lawrence (1).

The anionic nature of hexachlorophene would also suggest a lesser antibacterial action in the presence of quaternary ammonium compounds. The problem of ascertaining the extent of inactivation, if any, was undertaken because of the possible usage of both hexachlorophene and quaternary ammonium compounds in items such as lotions, powders, and creams. The methods selected for bacteriological evaluation were those commonly associated with the *in vitro* evaluation

Received January 4, 1962, from Sindar Corp., Delawanna, N. J.
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of these materials; namely, the zone of inhibition type of test and the broth tube serial dilution technique.

EXPERIMENTAL

Alcoholic stock solutions of benzalkonium chloride (alkylbenzyldimethylammonium chloride) and of hexachlorophene were mixed to give varying ratios of the active materials. In addition to these mixtures, equimolar amounts of hexachlorophene and benzalkonium chloride were brought to reaction in the following manner: To a solution of 8.1 Gm. of hexachlorophene in 100 ml. of acetone was added 15 Gm. of benzalkonium chloride (50% aqueous solution). The mixture was refluxed for 1 hour, the acetone distilled, and the solid removed by filtration, followed by drying at 50° in a 4-mm. vacuum. A slightly sticky material (15.5 Gm.) was obtained. The alkyl group in benzalkonium chloride represents a mixture of the alkyls C_8H_{17} to $C_{18}H_{37}$, the average being about $C_{13}H_{27}$, therefore the molecular weight of benzalkonium chloride approximates 354. The complex obtained from benzalkonium chloride and hexachlorophene would be about 724 (one mole of hydrogen chloride being removed) and its chlorine content 29.4%. The analysis of the product described above gave 29.5% chlorine.

For the bacteriological evaluation, aliquots of the alcoholic mixtures were added to tryptic soy broth (Difco) followed by twofold serial dilution in additional broth. Broth tubes were inoculated with one drop of a 1:100 water dilution of a 24-hour A.O.A.C. broth culture of *Staphylococcus aureus* A.T.C.C. 6538 (2). Tubes were incubated at 34° and growth was recorded by turbidity after 4 days. A slight haze was observed to form when alcoholic aliquots were added to the first tube of the serial dilution series. It was thought that this haze formation might lead to excess variation in the test and obscure any small differences in end points that might occur. For this reason, determinations were replicated seven times, each test being performed on a different day. The log means were analyzed for a significant difference from that of the hexachlorophene mean. It may be noted from Table I that, while both components of the mixture were highly bacteriostatic *per se*, a maximum loss in activity was approached as the ratio of components approached one to one.

An alternate approach for demonstrating the observed loss in activity was performed with the aid of 13-mm. filter paper disks (Schleicher & Schuell No. 740-E) which were impregnated with 0.07 ml. of alcoholic solutions of varying levels of hexachlorophene and benzalkonium chloride. The disks were dried at room temperature to remove solvent and then placed on seeded *S. aureus* plates. Replicate disks were prepared for each solution and placed on assay plates in a random manner. The plates were prepared such that each plate (100 mm. diameter) contained a 20-ml. base layer and a 6-ml. seed layer of agar. Dextrose tryptone extract agar (Difco) was employed for both the base layer and the seed layer. The inoculum consisted of a 1% (v/v) 24-hour A.O.A.C. broth culture of *S. aureus* in agar used for the seed layer. Sharp, well-defined inhibition zones were obtained for both hexachlorophene and benzalkonium chloride by this technique. The loss of zone production by mixtures of the active materials may be noted in Fig. 1. It may be observed that maximum loss of activity was

TABLE I.—BACTERIOSTATIC LEVELS OF MIXTURES OF HEXACHLOROPHENE AND BENZALKONIUM CHLORIDE AGAINST *Staphylococcus aureus*

	Bacteriostatic Levels (mcg./ml.) in Broth of Mixtures of the Following Composition											
% Benzalkonium Chloride	100	90	80	70	60	50	40	30	20	10	0	
% Hexachlorophene	0	10	20	30	40	50	60	70	80	90	100	Complex
Run No. 1	0.39	0.78	0.78	0.78	3.12	3.12	3.12	1.56	1.56	0.78	0.78	3.12
2	0.39	0.78	0.78	1.56	3.12	6.25	6.25	3.12	1.56	0.78	0.39	6.25
3	3.12	0.39	1.56	0.78	1.56	3.12	3.12	0.78	1.56	0.39	0.39	1.56
4	0.78	0.78	0.78	1.56	3.12	6.25	3.12	0.78	1.56	0.78	0.39	6.25
5	3.12	3.12	6.25	12.50	12.50	12.50	1.56	0.78	0.78	0.39	0.78	3.12
6	0.39	0.39	0.39	0.78	1.56	6.25	3.12	3.12	3.12	0.39	1.56	6.25
7	0.39	0.78	1.56	1.56	0.78	1.56	1.56	1.56	1.56	0.78	1.56	12.50
Geometric mean	0.78	0.78	1.16	1.56	2.56	4.60	2.74	1.42	1.56	0.59	0.71	4.65
t^a	...	...	...	1.56	2.75	4.88	4.19	1.85	...	...	...	4.72

[a] Value of t at the 0.05 level of significance for 12 degrees of freedom is 2.18 (3).

TABLE II.—ZONE PRODUCTION BY EQUIMOLAR MIXTURES OF QUATERNARY AMMONIUM COMPOUNDS AND HEXACHLOROPHENE AGAINST *Staphylococcus aureus*

Quaternary Ammonium Compound	Concentration of Components of Mixture, mcg./ml.		Inhibition Zone,[e] mm.		
	Hexachlorophene	Q.A.C.[d]	Hexachlorophene	Q.A.C.	Equimolar Mixture
Hyamine 1622[a]	2500	2750	5.3	1.2	1.7
Hyamine 10-X[b]	2500	2580	5.3	1.1	0.5
Hyamine 2389[c]	2500	2250	5.9	2.0	0.6
Cetylpyridinium chloride	2500	2070	5.8	0.4	0.3

[a] Hyamine 1622, (Rohm & Haas Co.) (diisobutylphenoxyethoxyethyl)benzyldimethylammonium chloride, monohydrate. [b] Hyamine 10-X (Rohm & Haas Co.) (diisobutylcresoxyethoxyethyl)benzyldimethylammonium chloride, monohydrate. [c] Hyamine 2389 (Rohm & Haas Co.) [alkyl(C9–C15)metnylbenzyl]trimethylammonium chloride. [d] Quaternary ammonium compounds. [e] Average zone of three replicates to nearest 0.1 mm.

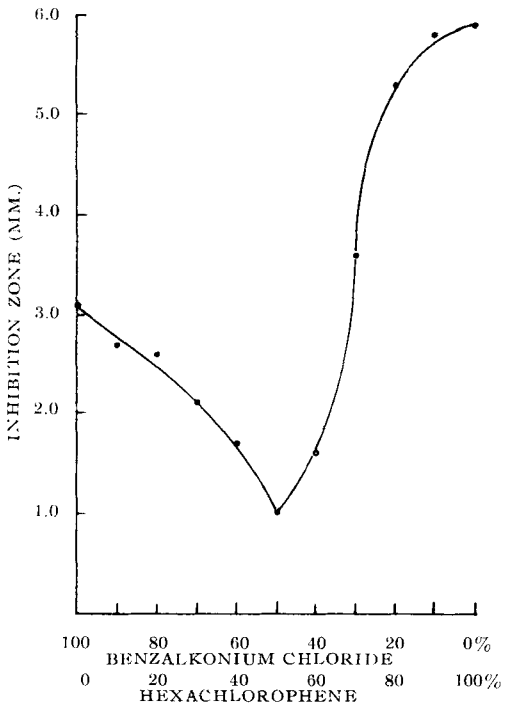


Fig. 1.—Effect of varying ratios of hexachlorophene and benzalkonium chloride on the inhibition zone produced against *S. aureus*. Each point is the mean of three determinations. Solutions were 0.1% with respect to total mixture present.

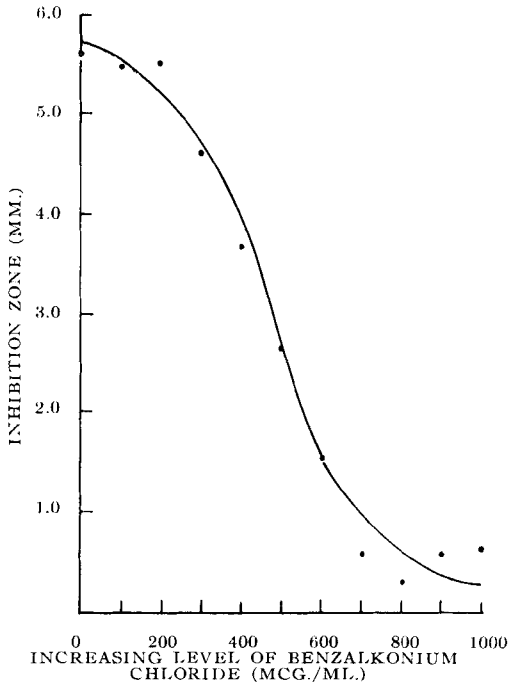


Fig. 2.—Diminishing zone production of a 1000 mcg./ml. hexachlorophene solution with increasing levels of benzalkonium chloride.

found at approximately a 1:1 ratio. Alcoholic mixtures of hexachlorophene and benzalkonium chloride, examined by the plate technique, in which the hexachlorophene level was held constant may be seen in Fig. 2. A loss of activity as the mixture approached a 1:1 ratio was observed.

Several quaternary ammonium compounds structurally dissimilar to benzalkonium chloride were examined in equimolar mixtures with hexachlorophene by the plate technique. As may be seen in Table II, zones produced by these mixtures were also considerably smaller than those of the active materials *per se*.

DISCUSSION AND SUMMARY

Bacteriological examination of mixtures of quaternary ammonium compounds and hexachlorophene demonstrated an antagonistic relationship when evaluated by tube dilution and agar plate techniques. The maximum loss of activity was observed at an approximate equimolar concentration of each component of the mixture. The loss of activity by broth dilution was less dramatic than on agar plates. The haze observed in the broth tubes during the conduct of the tests indicates the formation of an insoluble reaction product. This loss of activity was greatly magnified in the plate technique which relies on the ability of materials to diffuse through agar.

Mixtures of hexachlorophene and quaternary ammonium compounds produce a relatively insoluble complex which is less active than the components, *per se*, of the mixture. The activity loss is greatest at an equimolar ratio, and it must be assumed that one is determining the activity of the uncombined excess of one or the other component when ratios other than equimolar are examined.

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Thioacetamide I

Substitute for Hydrogen Sulfide in Medicinal Chemicals for Identity and Purity

By JACK JUE and C. LEE HUYCK†

Identification tests and tests for heavy metals in inorganic chemicals using thioacetamide solution and hydrogen sulfide solution gave identical results. Difficulty was encountered only when solutions of organic drugs in volatile flammable solvents interfered with the hydrolysis of thioacetamide. In this case, the length of time for the completion of the test was increased in order to insure complete hydrolysis of the thioacetamide. In testing acetylsalicylic acid, methenamine, and salicylic acid contaminated with the minimum amount of solution of lead salt sensitive to hydrogen sulfide test solution, the color of the precipitate was brown instead of black.

THE PURPOSE of this study is to review the previous work done on the use of thioacetamide test solution as a substitute for hydrogen sulfide test solution in identifying salts of heavy metals, to detect heavy metal impurities in chemicals, and to investigate the possibility of using thioacetamide test solution as a substitute for hydrogen sulfide test solution.

Hydrogen sulfide from generators or compressed hydrogen sulfide is troublesome compared with aqueous solutions of a solid compound such as thioacetamide as a source of the gas. An added disadvantage of hydrogen sulfide is unpleasant odor, with both systemic and local toxicity.

Thioacetamide (1) is available in the form of white crystals. It is soluble in water, alcohol, benzene, and sparingly soluble in ether. Its aqueous solution is neutral and stable at room temperature. A slight sediment may develop upon standing, but the liquid still has precipitating power. In solution, thioacetamide is a mixture of the amide and its enol form in equilibrium

$$CH_3CSNH_2 \leftrightarrows CH_3C(SH)=(NH)$$

Hydrolysis occurs in both alkaline and acid solutions. Both acids and bases catalyze the hydrolysis. The equations for the reactions are as follows

with acids: $CH_3CSNH_2 + 2H_2O \rightarrow CH_3COONH_4 + H_2S$

with bases: $CH_3CSNH_2 + 3OH^- \rightarrow CH_3COO^- + NH_3 + S^{2-}$

The hydrolysis reaction may be expressed as follows

$$CH_3CSNH_2 + H_2O \rightarrow H_2S + CH_3CONH_2$$

Thioacetamide hydrolyzes slowly in the cold but more rapidly at elevated temperature; therefore, the rate of hydrolysis can be controlled by regulating the temperature. The thioacetamide is introduced into the solution to be analyzed and is heated to approximately 80° on a water bath to cause hydrolysis. Hydrogen sulfide is liberated, causing metal sulfides to be precipitated. The precipitates formed with thioacetamide are coarser and more easily separated by centrifuging or filtering than those formed with hydrogen sulfide. The cations are precipitated in nearly stoichiometric amounts and very little of the hydrogen sulfide ever escapes into the air. There is no need for delivery tubes, safety traps, or hoods. The thioacetamide is usually used in the form of a 10% aqueous solution, the most common being 1*M* or 7.5% w/v concentration.

Vavilov (2) showed that bismuth can be detected in the presence of metals of group IV with thioacetamide. When a drop of bismuth solution is treated with an alcoholic solution of thioacetamide, a yellow coloration is produced. It is probable that $(BiO).SCNHCH_3$ is formed as a result of the reaction between the univalent BiO and the thioacetamide. Cations of group III and copper show evidence of behaving similarly.

Barber and Grzeshowiak (3) used thioacetamide in place of gaseous hydrogen sulfide for precipitation of insoluble sulfides. Thioacetamide was sucessfully used in place of gaseous hydrogen sulfide in the development of new methods of qualitative analysis of group II and group III. A water solution of thioacetamide was added directly to the solution of cations. No great ex-

Received December 8, 1961 from the St. Louis College of Pharmacy, St. Louis, Mo.
Accepted for publication January 15, 1962.
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cess of thioacetamide was required. Nearly equivalent amounts of the organic sulfide and the cation brought about complete precipitation of the insoluble sulfide. The hydrolysis of thioacetamide gave a relatively low concentration of the sulfide ions favoring the rapid coagulation and filtration of the insoluble sulfides. The time required for complete precipitation of the insoluble sulfides by thioactamide was less than the overall time required when gaseous hydrogen sulfide was used. The advantages of thioacetamide solution are tolerable odor, good stability, low cost, and a high degree of solubility in water.

Fales and Kenny (4) gave alternative thioacetamide precipitation procedures which have been used for the last 3 years in our classes.

Bloemendal and Veerkamp (5) found that thioacetamide can fully replace hydrogen sulfide and ammonium sulfide in the qualitative test for heavy metals. A simple analysis procedure was given. They also found that other sulfide compounds like thioformamide are less stable, but usable. Thiourea, thioglycollate, thioacetate, thiosalicylate, and thiophosphate, however, are useless as a replacement for hydrogen sulfide.

Flaschka (6) and Flaschka and Abdine (7) made a study of the use of thioacetamide. Qualitative procedures using thioacetamide were fundamentally identical with those using hydrogen sulfide and differ only in manipulative details. Some precipitation and separation procedures were outlined.

Flaschka and Abdine (7) were among the first to study thioacetamide in quantitative analysis. They found that since thioacetamide formed coarser precipitates, the precipitates are less contaminated by foreign ions, easier to filter, and therefore more suitable for gravimetric analysis than those formed by hydrogen sulfide.

Flaschka and Abdine (7, 8) studied some thioacetamide separations in the presence of ethylenediaminetetraacetic acid (EDTA). The EDTA prevented the precipitation of some metals as their sulfides through chelation. Equilibrium of the metal-EDTA-thioacetamide system resulted by the addition of the calcium ion. Metals were separated as sulfides by precipitating some with thioacetamide in EDTA solution and separating the remainder by the addition of a large amount of calcium ions.

Amin (9) used thioacetamide with EDTA titrations in complexometric analysis of different ores and alloys. Also thioacetamide was used with ion-exchange resins in the analysis of various ores.

Swift and Butler (10) do not agree that thioacetamide solutions can be substituted for hydrogen sulfide without modification of procedure. Rate of reaction measurements have shown that the hydrolysis of thioacetamide to hydrogen sulfide and acetamide is a first-order reaction with respect to both thioacetamide and hydrogen ion concentration, and only a small fraction of the acetamide formed is hydrolyzed to acetic acid and ammonia. In solutions having pH values of less than 3, the precipitation of lead sulfide by thioacetamide is preceded by hydrolysis and the rate of precipitation equaled the rate of hydrolysis. In solutions having pH values from 3.5 to 5.1, the precipitation is first order with respect to both thioacetamide and lead ion concentration. At pH 5, the rate of precipitation of lead sulfide with hydrogen sulfide was found to be 1000 times greater than the formation of lead sulfide by hydrolysis of thioacetamide.

Monte Bovi (11) who first suggested the use of thioacetamide in the testing of pharmaceuticals found it to be a satisfactory replacement for hydrogen sulfide in certain tests used to establish the identity of mercury, bismuth, zinc, and manganese salts, and to detect the presence of such impurities as mercury (II) in merbromin and nitromersol, mercuric iodide in yellow mercurous iodide, copper or zinc in methylene blue, iron in zinc oxide, and heavy metals in sodium chloride and potassium nitrate. Monte Bovi concluded "there is need for more fundamental studies, especially on a quantitative basis, to establish the sensitivity of thioacetamide and the mechanism of reaction, before it can be adopted as a direct replacement for hydrogen sulfide in all pharmaceutical testing. Until then, the indiscriminate use of thioacetamide as a replacement for hydrogen sulfide is not justified."

EXPERIMENTAL

Every identification test for heavy metal salts and every test for heavy metal impurities in chemicals of the U.S.P. (12) was performed with both hydrogen sulfide test solution and thioacetamide aqueous solution. The hydrogen sulfide test solution was made by passing an excess of hydrogen sulfide into cold distilled water. The solution was stored in a small, dark amber-colored bottle filled nearly to the top and kept in a cool dark place. It was not used unless it possessed a strong odor of hydrogen sulfide and unless it produced a copious precipitate with an equal volume of ferric chloride test solution.

The thioacetamide was 1 *M* or 7.5% w/v. The commercial reagent grade thioacetamide was used. The solution was stored in a dark amber-colored bottle similar to the bottle used to store the hydrogen sulfide test solution. An equal volume of this solution, previously heated on a water bath to approximately 80° for a minimum of 5 minutes (in order to hasten the hydrolysis of the thioacetamide) was

used along with the hydrogen sulfide solution in all the tests for comparative purposes.

In the tests for identification, the procedure of the U.S.P. was followed with both solutions. In testing for heavy metals, contamination was accomplished by addition of 0.001 mg. lead in the form of a water-soluble salt dissolved in 1 ml. distilled water. This amount of lead is the minimum amount that gives a color with hydrogen sulfide test solution and was the standard of comparison for the thioacetamide solution.

Where applicable, official tests for identification were conducted as directed in the U.S.P. with the two solutions, and no difference in color or appearance could be detected. See Table I.

TABLE I.—CHEMICALS WHICH YIELDED EQUALLY POSITIVE IDENTIFICATION TESTS WITH HYDROGEN SULFIDE AND THIOACETAMIDE TEST SOLUTIONS

Antimony potassium tartrate	Mercuric chloride
Antimony sodium thioglycolate	Yellow mercuric oxide
Bismuth potassium tartrate	Mersalyl
Bismuth subcarbonate	Zinc peroxide
Bismuth subsalicylate	Zinc stearate
Carbarsone	Zinc sulfate

Tests for heavy metals impurities were conducted as directed with the two solutions, after contaminating them with lead as directed above. Differences were noted with acetylsalicylic acid, methenamine, and salicylic acid. Solutions of these chemicals required a slight amount of heat in addition to the heat supplied by the reagent. After warming slightly on a water bath, instead of a black precipitate, a brown precipitate was observed. No differences in color or appearance could be detected when the two solutions were used in testing the chemicals contaminated with heavy metals. See Table II.

TABLE II.—CHEMICALS CONTAMINATED WITH HEAVY METALS WHICH YIELDED EQUALLY POSITIVE TESTS WITH HYDROGEN SULFIDE AND THIOACETAMIDE TEST SOLUTIONS

Aluminum sulfate	Magnesium oxide, heavy
Ammonium carbonate	Magnesium sulfate
Ammonium chloride	Magnesium trisilicate
Barium sulfate	Potassium bicarbonate
Boric acid	Potassium bromide
Calcium carbonate	Potassium chloride
Calcium chloride	Potassium hydroxide
Calcium gluconate	Potassium iodide
Calcium hydroxide	Potassium nitrate
Calcium lactate	Sodium bicarbonate
Calcium mandelate	Sodium biphosphate
Dibasic calcium phosphate	Sodium bisulfate
Ferrous sulfate	Sodium borate
Diluted hydriodic acid	Sodium bromide
Hydrochloric acid	Sodium chloride
Diluted hydrochloric acid	Sodium citrate
Hydrogen peroxide solution	Sodium iodide
	Sodium phosphate
Magnesium carbonate	Sodium salicylate
Magnesium oxide	Sodium sulfate

Likewise, U.S.P. organic chemicals examined for heavy metals were contaminated with a lead solution as previously described and tested with the two solutions as directed. No difference in color or appearance could be detected. See Table III.

TABLE III.—ORGANIC CHEMICALS CONTAMINATED WITH HEAVY METALS WHICH YIELDED EQUALLY POSITIVE TESTS WITH HYDROGEN SULFIDE AND THIOACETAMIDE TEST SOLUTIONS

Amylene hydrate	*p*-Aminobenzoic acid
Barbital sodium	Pentobarbital sodium
Carbachol	Phenobarbital sodium
Citric acid	Phenolphthalein
Dextrose	Propylene glycol
Ethyl aminobenzoate	Saccharin
Gelatin	Sodium saccharin
Glucose	Sucrose
Glycerin	Tartaric acid
Iodoalphionic acid	Theobromine calcium salicylate
Lactic acid	
Lactose	Thiopental sodium
Methyl salicylate	Urea
Nicotinamide	Urethane
Nicotinic acid	

In addition, the official pharmaceuticals and their preparations were contaminated with lead solution and tested for heavy metals by the two solutions as previously described. No difference in color or appearance could be detected. Pharmaceuticals and preparations tested are given in Table IV.

TABLE IV.—PHARMACEUTICALS CONTAMINATED WITH HEAVY METALS WHICH YIELDED EQUALLY POSITIVE TESTS WITH HYDROGEN SULFIDE AND THIOACETAMIDE TEST SOLUTIONS

Aluminum acetate solution	Ammoniated mercury ointment
Aluminum hydroxide gel	Orange flower water
Aluminum phosphate gel	Orange oil
Diluted ammonia solution	Ringer's solution
Caffeine and sodium benzoate	Lactated Ringer's solution
Cinnamon oil	Stronger rose water
Clove oil	Sassafras oil
Dextrose injection	Isotonic sodium chloride solution
Milk of magnesia	Distilled water
Zinc peroxide	

DISCUSSION OF RESULTS

Identification tests for heavy metals showed that thioacetamide test solution and hydrogen sulfide test solution, with few exceptions, gave identical results. Difficulty was encountered in testing water-insoluble organic salts for heavy metals because the volatile flammable solvents used had to be heated. In this case, the length of time of standing on a hot water bath could be increased in order to insure the complete hydrolysis of thioacetamide.

Plans are made to compare thioacetamide with hydrogen sulfide in the identification and detection of heavy metals in chemicals and pharmaceuticals

of the National Formulary. Comparative quantitative analyses with thioacetamide and hydrogen sulfide are also planned for chemicals of both the United States Pharmacopeia and the National Formulary where hydrogen sulfide is directed as the quantitative precipitating reagent.

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Kinetics of Reaction between Molecular Oxygen and 2,3-Dimercapto-1-propanol in Aqueous Solution

By EDWARD G. RIPPIE† and TAKERU HIGUCHI

Autoxidation of BAL is shown to take place in the absence of metallic catalysts. Although the iodometric sulfhydryl concentration in this system was found to decrease linearly with time, the reaction was found to approach true zero order only at higher initial BAL concentration. The observed rate of oxidation was found to decrease with increased hydrogen ion concentration and to be of a mixed order with respect to oxygen tension.

BARRON, *et al.* (1), have suggested that the observed reaction in aqueous solution between 2,3-dimercapto-1-propanol, BAL, and molecular oxygen in absence of added catalyst could be entirely attributed to trace quantities of catalytic copper. In this communication, experimental evidence which supports the existence of the uncatalyzed reaction and kinetic dependencies of this oxidative reaction on pH, oxygen tension, and initial substrate concentration are presented.

EXPERIMENTAL

Reagents and apparatus employed in the work were the same as those reported in previous work on the copper catalyzed oxidation of BAL (2). The procedure used was also essentially the same except that potassium cyanide was added to all reaction solutions at a concentration of $1 \times 10^{-3} M$. While catalase is known to be inhibited by cyanide (3), sufficient catalase was found to remain to destroy satisfactorily any peroxide which may have been formed. The reacting systems were sufficiently stirred (2) so that the solutions were always kept in equilibrium with the gas phase.

Received October 13, 1961, from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication November 20, 1961.
This study was supported in part by a grant from Parke, Davis & Co., Detroit, Mich., and in part by the Research Committee of the Graduate School from funds furnished by the Wisconsin Alumni Research Foundation.
† University of Wisconsin Fellow 1958–1959. Present address: College of Pharmacy, University of Minnesota, Minneapolis.

RESULTS AND DISCUSSION

Evidence for Uncatalyzed Reaction.—There appears to be ample evidence which points to reaction in the absence of copper between molecular oxygen and BAL in aqueous solution. As has already been shown (2), significant copper concentration, *ca.* $2 \times 10^{-6} M$, is apparently necessary to account for the observed rate. If such high concentrations of the metal were introduced by chance contamination, it would be expected that variation in sources of buffer salts, water, and substrate would produce significant changes in the observed rates of oxidation. This was not the case. Quite reproducible rates were observed for different lots of buffer salts, BAL, and water.

Further evidence supporting the existence of uncatalyzed reaction is presented in Fig. 1. These plots show the residual sulfhydryl concentration of aqueous solutions of BAL as a function of time when exposed to oxygen at 1 atm. The uppermost curve shows the kinetic dependency for a system free of copper and cyanide as far as was possible experimentally. The second line shows essentially the same system but with $1 \times 10^{-3} M$ potassium cyanide added. It is evident that these two functions are quite similar, both qualitatively and quantitatively. The third and fourth curves show the effects of addition of copper, 2×10^{-6} and $5 \times 10^{-6} M$, respectively, on the initial system. The kinetic behavior of these last two runs is obviously quite different from those of the preceding two. Although the data are not shown, addition of $1 \times 10^{-3} M$ cyanide to the latter two systems yielded rate curves identical with the first two.

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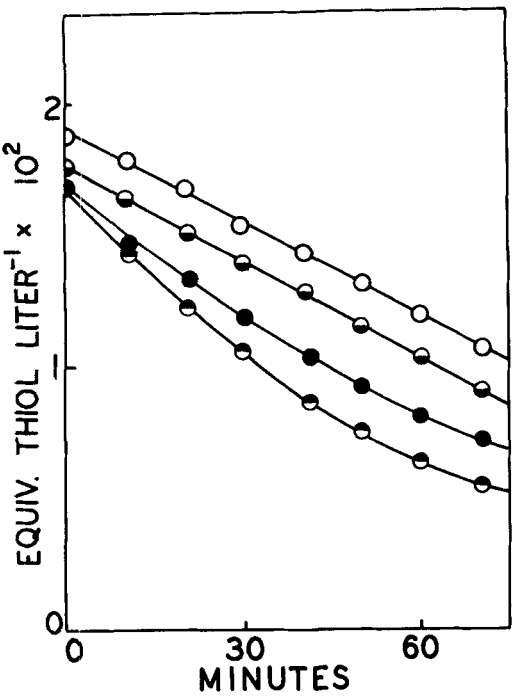


Fig. 1.—Plots contrasting BAL dependency of the oxidation with and without copper. ○, essentially copper free; ◒, previous system plus 0.001 *M* cyanide; ●, initial system plus 2 × 10^{-6} *M* copper; ◓, initial system plus 5 × 10^{-6} *M* copper; temp., 20°; pH, 6.66; 0.25 *M* phosphate buffer.

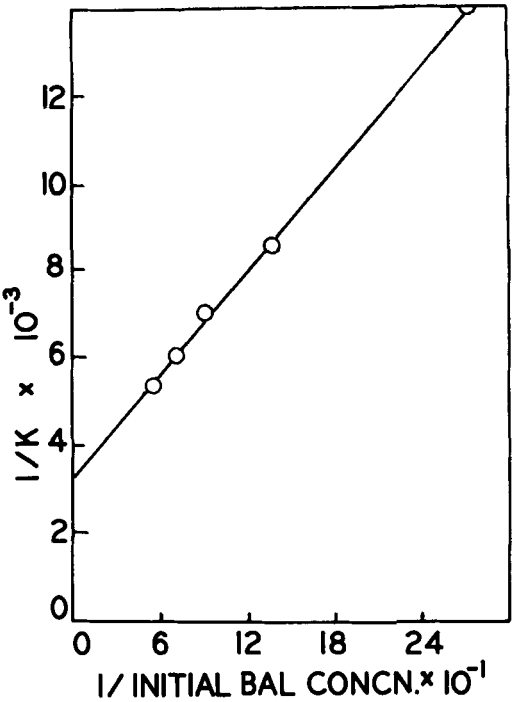


Fig. 2.—Reciprocal zero-order rate constants plotted against the reciprocal initial BAL concentration. The extrapolated value of the limiting rate is 3.06 × 10^{-4} equiv. per L. per min.; temp., 20°; pH, 6.66; 0.25 *M* phosphate buffer; 1 atm. oxygen.

These facts strongly suggest that BAL can undergo oxidation by molecular oxygen in the absence of copper or any other metallic catalyst. Any catalytic contribution from trace copper appears to be removed effectively by addition of cyanide. Although the part played by the uncatalyzed reaction would be relatively small in the presence of certain metallic ions, it would appear that for simple aqueous systems this would be the important kinetic route of oxidation of the sulfhydryl compound.

Order of the Reaction with Respect to BAL.—The behavior of BAL concentration with respect to time as shown in the upper two plots of Fig. 1 is typical of the uncatalyzed reaction. Essentially straight-line decrease is experienced although the absence of a short initial lag time could not be unequivocally established since readings were difficult to obtain during the first few minutes. Determinations of apparent zero-order rate constants were made over a temperature range of 0 to 24.5° at pH values from 6.50 to 7.14. Individual runs were made with initial molar BAL concentrations of 0.0037, 0.0074, 0.0111, 0.0148, and 0.0185. The apparent zero-order disappearance which was observed through approximately 75 per cent of the reaction suggests a rather complex dependency on the dithiol.

Although individual runs followed apparent zero-order kinetics, marked differences in the rates were found when varying initial concentrations were employed. When the reciprocals of these apparent zero-order rate constants were plotted against the reciprocals of the initial BAL concentrations, a

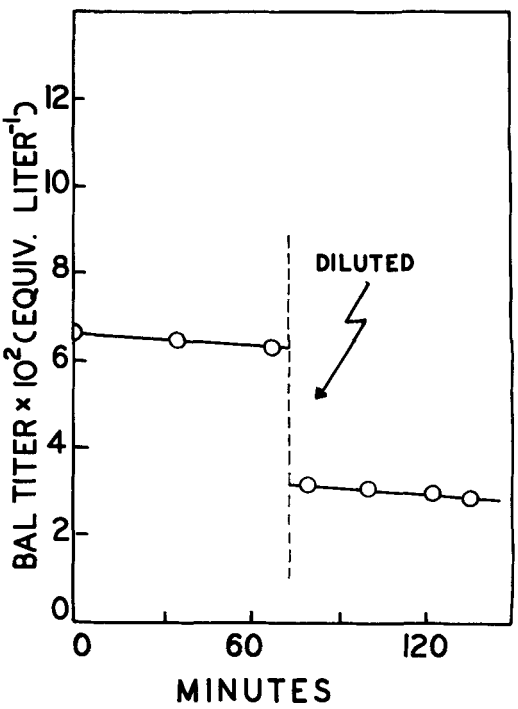


Fig. 3.—Plot of BAL concentration against time showing the effect of dilution of the reaction mixture at a time indicated by the dotted line. Temperature, 0°; 2 atm. oxygen; pH, 6.66; 0.25 *M* phosphate buffer.

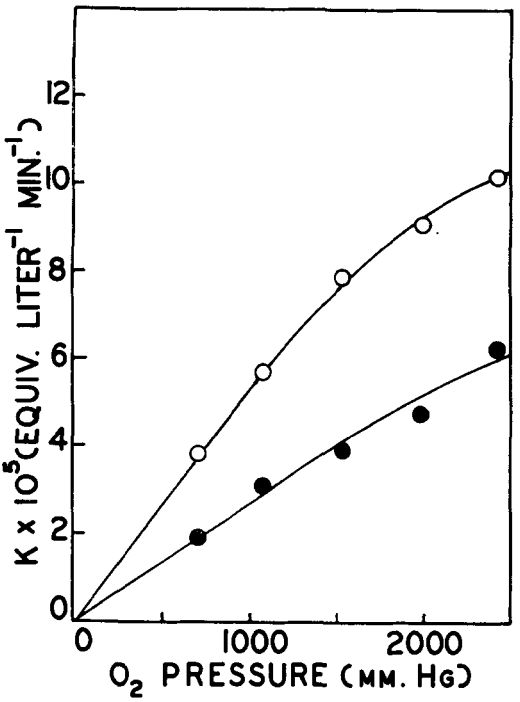


Fig. 4.—Plots of zero-order rate constants of BAL oxidation against oxygen pressure. Initial BAL concentrations: ○, 0.0370; ●, 0.0185; temp., 0°; pH, 6.66; 0.25 *M* phosphate buffer.

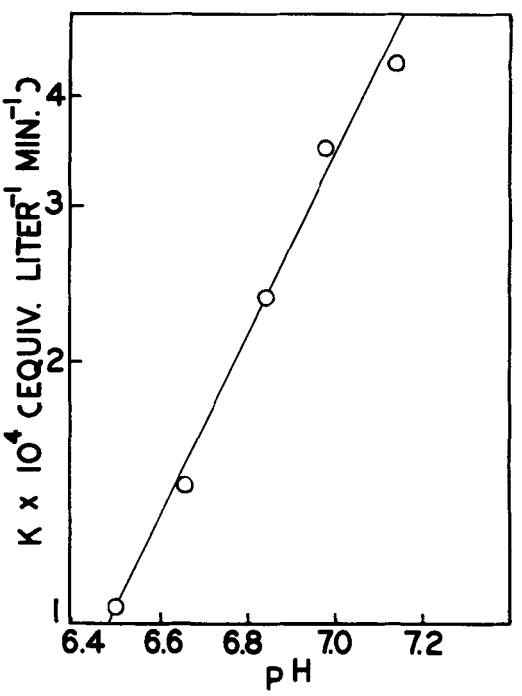


Fig. 6.—Semilog plot of zero-order rate constant against pH determined at 20°. Slope of the line is 1.0; 1 atm. oxygen; 0.25 *M* phosphate buffer.

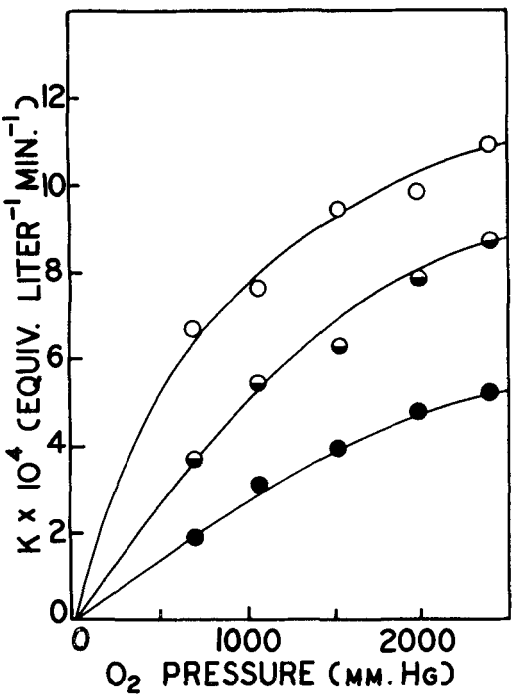


Fig. 5.—Plots of zero-order rate constants of BAL oxidation against oxygen pressure. Initial BAL concentrations; ○, 0.0370; ◒, 0.0185; ●, 0.0093; temp., 24.5°; pH, 6.66; 0.25 *M* phosphate buffer.

straight line plot was obtained, as shown in Fig. 2. The intersection of this line with the vertical axis above the origin appears to indicate a finite limiting rate of 3.06×10^{-4} equiv. per L. per min. at theoretically infinite BAL concentration.

Runs were made in which the reaction was allowed to proceed for several minutes, after which the reaction mixture was diluted with an equal volume of solvent solution. A typical run plotted in Fig. 3 shows a change in oxidation rate upon dilution. The rate of oxidation after dilution corresponds to the expected rate, had the reaction been started at half the initial BAL concentration. These observations serve to show that the reaction is truly zero order with respect to BAL only at very high concentrations. Apparent zero-order kinetic behavior at lower concentrations suggests that a rather complex situation exists.

Oxygen Dependency.—Determination of oxygen dependency was carried out at a pH of 6.66, showing oxidation rate as a function of oxygen tension at 0 and 24.5°. Oxygen pressures ranged from 1 to 3 atm. and rate constants are given for initial BAL concentrations of from 0.0093 to 0.0370 *M*. The results of these determinations are plotted in Figs. 4 and 5. Although the oxidation rate appears to be first order with respect to oxygen tension at low oxygen pressures, the dependency at higher oxygen pressures appears to decrease. The rate, however, does not appear to approach a finite limiting value at high oxygen pressures.

***Effect of* Hydrogen Ion Concentration.**—The effect of pH on the rate of BAL autoxidation was determined between pH 6.4 and 7.2 at an initial BAL concentration of 0.0111 *M*. In Fig. 6 the logarithm of the rate in equivalents per L. per min.

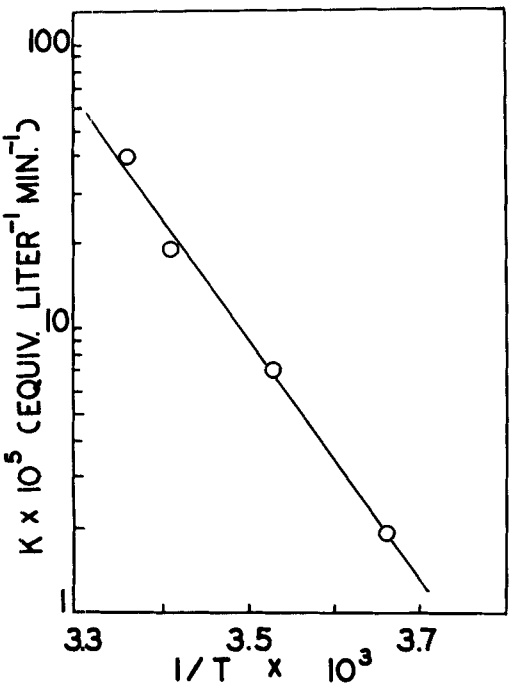


Fig. 7.—Arrhenius plot of zero-order rate constants against reciprocal absolute temperature. Determinations made at pH 6.66 and a pressure of 1 atm. oxygen; 0.25 *M* phosphate buffer.

has been plotted against the pH of solutions buffered by 0.25 *M* potassium phosphate. The isotherm has a positive slope of 1.0, showing more rapid oxidation at the higher pH.

Temperature Dependence.—A semilog plot of apparent zero-order rate constants determined at 0, 10, 20, and 24.5° *vs.* reciprocal absolute temperature is shown in Fig. 7. The heat of activation, ΔHa, as calculated from the slope, was 20.1 Kcal. per mole. Since the effect of temperature on oxygen solubility or on the degree of ionization of BAL was not taken into account, the heat of activation given is the apparent heat of activation. This can be corrected by subtracting the heat of solution of oxygen in water (−3.6 Kcal. per mole) to give a corrected ΔHa of 23.7 Kcal. per mole. The heat of ionization of BAL, however, was not known and no correction has been made for its presumably small effect. The ΔHa found here was considerably larger than that found for the copper catalyzed oxidation (16.2 Kcal. per mole).

Consideration of the Overall Reaction.—It is evident from the dependency on BAL and oxygen that the reaction mechanism encountered here is probably quite complex. It was suggested by Harrison (4) that the disulfide linkage is essential in the autoxidation of sulfhydryl compounds. Highly reactive cyclic disulfide may possibly play an important role in BAL oxidation, acting as a source of the radical. The 23.7 Kcal. per mole activation energy found for the overall process is of the magnitude which might be expected for the homolytic cleavage of the disulfide bond (5) of the oxidized BAL monomer. The possibility of heterogeneous catalysis by the glass wall of the reaction vessel was investigated and ruled out.

The overall mechanism which fits the observed facts can be formulated in any number of ways. The apparent linear decrease of sulfhydryl concentration with time may be rationalized, for example, on the basis that a significant buildup of reactive cyclic disulfide may occur. Another rationalization may be based on the assumption that two BAL molecules linked by a disulfide bond may undergo autoxidation more readily than BAL itself. The obvious zero-order character of the reaction with respect to BAL at high BAL concentration appears to suggest, on the other hand, that the dithiol itself may act as an efficient chain stopper at higher concentrations. Such information as is presently available appears to be insufficient to resolve the question.

The following series of reactions may possibly play a part in the oxidative breakdown of BAL in the absence of copper

$$B(SH)S^- + BS_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B(SH)S\cdot +$$

$$BS_2^{-}\cdot \overset{H^+}{\rightleftharpoons} 2\ B(SH)S\cdot$$

$$B(SH)S\cdot + O_2 \xrightarrow{k_2} BS_2 + HO_2\cdot$$

$$HO_2\cdot + B(SH)S^- \xrightarrow{k_3} HO_2^- + B(SH)S\cdot$$

$$BS_2 \xrightarrow{k_4} (BS_2)_x \text{ polymer}$$

It offers an explanation of the dilution effect noted previously and the fact that the rate depends upon the initial BAL concentration. Other reactions no doubt take place (6) since this series of equations does not predict mixed-order kinetics with respect to oxygen at higher pressures. It should be emphasized that the chemical reactions suggested here are presented as those which may occur. Because of the apparent complexity of the findings, the overall process in reality may consist of many interacting pathways.

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Barbiturate Glucosides

By GLENN A. PORTMANN† and LOYD E. HARRIS

Nitrogen barbiturate glucosides of barbital, phenobarbital, amobarbital, secobarbital, and pentothal were prepared from acetobromoglucose and the potassium barbiturate. Proof of the nitrogen glucosidic linkages was obtained by direct condensation of sym-di-(tetra-O-acetyl-β-D-glucopyranosyl)urea with diethylmalonyldichloride. The lack of hypnotic activity of the barbiturate glucosides was demonstrated with intraperitoneal injections of 6 to 25 times the hypnotic dose of the corresponding barbiturate salt.

IT IS WELL KNOWN that many glycosides have very pronounced and widely varied pharmacological effects. The type of pharmacological response always depends on the specific structure of the aglycone but the sugar moiety contributes important absorption and distribution characteristics. However, a survey of the literature pertaining to glucosides of pharmacologically active compounds showed no consistent change in the varied responses of the compounds studied.

Many investigators have studied the effect of glucose and its metabolic products on barbiturate anesthesia. Lamson and his co-workers (1) were the first to publish their observations made when glucose was injected into dogs which recovered from pentobarbital anesthesia. They found that the dogs returned to anesthesia and remained in this state for an average time of 1 hour. Upon subsequent injections of glucose, the duration of anesthesia decreased until eventually further anesthesia could not be produced. Some other substances which will also cause this return to barbiturate anesthesia are products of glycolysis and the Krebs cycle, vitamins, malonic acid, glycerin, and epinephrine (2–5). Bester and Nelson (6) found that normal blood glucose levels did not affect pentobarbital anesthesia and proposed a competitive inhibition mechanism to explain the significant increase in sleeping time when glucose was injected with pentobarbital. Further evidence supporting an oxidative inhibitory mechanism is animal protective studies with cytochrome C injected with pentothal (7) and the need for greater quantities of pentothal to produce anesthesia in insulin-treated animals (8).

The present study was undertaken in order to determine whether glucose would have a potentiating effect if chemically combined with barbiturates. Furthermore, stable water-soluble barbiturate glucosides would be advantageous for liquid pharmaceutical dosage forms.

Only three barbiturate glycosides are reported in the literature. The mono-glucoside of barbital was prepared by reacting diethylmalonyldichloride with tetra-O-acetylglucose-urea (9). Bodendorf (10) prepared 5,5-diethyl-1,3-di-(tetra-O-acetyl-β-D-glucopyranosyl)barbituric acid from tetra-O-acetyl-α-D-glucopyranosylbromide and potassium barbital with subsequent deacetylation by sodium methoxide. Only one enolic barbiturate glucoside has been prepared utilizing the enolizing influence of silver oxide. Snyder and Link (11) prepared 5,5-diethyl-4-(tetra-O-acetyl-β-D-glucopyranosyloxy)-2,6-pyrimidinedione in 2.6% yield from 5,5-diethylbarbituric acid and tetra-O-acetyl-α-D-glucopyranosylbromide in the presence of silver oxide and a catalytic amount of quinoline.

EXPERIMENTAL[1]

Tetra-O-acetyl-α-D-glucopyranosylbromide.—Tetra-O-acetyl-α-D-glucopyranosylbromide, commonly known as acetobromoglucose, was prepared by a combination of two known methods (12, 13). Two hundred grams of β-D-glucose pentaacetate prepared by a standard procedure (14) was mixed with 350 ml. of 30% hydrogen bromide in glacial acetic acid and kept at room temperature for 4 hours. Hydrogen bromide was removed under reduced pressure at 40–50°, passing dry nitrogen through the capillary. Excess acetic acid was distilled at the bath temperature of 55–60° and 15 mm. Hg. The dark brown residue was crystallized twice from dry ether giving white needles; yield 127 Gm. (60%), m.p. 89–90°.

Glucosides of Barbital, Phenobarbital, Amobarbital, and Secobarbital.—These four nitrogen glucosides were prepared by following general procedure. Fifteen milliliters of 2 *N* potassium hydroxide was added to 50 ml. of an acetone solution containing acetobromoglucose (0.030 mole) and the barbiturate (0.015 mole). After 4 days reaction time, the absence of acetobromoglucose was shown by a negative silver nitrate test. The acetone was then evaporated and the resulting syrup was dissolved in 50

Received October 19, 1961, from the Research Dept., White Laboratories, Inc., Kenilworth, N. J.
Accepted for publication November 3, 1961.
Abstracted in part from the dissertation presented to the Graduate School, Ohio State University, for the degree of Doctor of Philosophy.
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[1] Melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected. Analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

ml. benzene and extracted with three 50-ml. portions of 20% sodium hydroxide, followed by water extraction until all the base was removed. After drying the benzene solution over anhydrous sodium sulfate, the solvent was evaporated giving hard, white, glassy products. Yields and physical constants are listed in Table I. Only the acetylated glucoside of barbital was crystallizable. Two crystallizations from absolute ethanol gave 1.8 Gm. (14%) of fine needles. None of the four acetylated nitrogen glucosides reduced hot Benedict's reagent.

Elemental analyses shown in Table I indicate that a di-glucoside was formed in every case except with amobarbital. Infrared spectra support this by the presence of imino group absorption at 3.1 μ with the acetylated amobarbital glucoside and the absence of this absorption in the spectra of phenobarbital, secobarbital, and barbital acetyl glucosides.

Deacetylations were done by partially dissolving the acetylated glucoside (0.002 mole) in 100 ml. of absolute ethanol, adding 6 ml. of sodium methoxide solution (1 mg. sodium per ml. of methanol) and distilling the methylacetate and alcohols. After decolorizing with charcoal, white hard amorphous compounds were obtained which were very water soluble but were not crystallizable. After acid hydrolysis, they reduced hot Benedict's reagent. Deacetylation of the crystalline barbital glucoside was also accomplished with ammonia in absolute methanol. The acetylated compound (0.0015 mole) was partially dissolved in 60 ml. of absolute methanol and saturated with dry ammonia gas at 5°. After 1 day, the ammonia and methanol were evaporated and the acetamide was removed from the white residue with 10 ml. of chloroform. The yield was 0.50 Gm. (0.0010 mole) of a white, brittle, amorphous solid.

Anal.—Calcd. for $C_{20}H_{32}N_2O_{13}$: N, 5.51. Found: N, 5.12.

5-Ethyl-5-(1-methylbutyl)-1,3-di-β-D-glucopyranosyl-2-thiobarbituric Acid.—Acetobromoglucose (8.2 Gm., 0.02 mole) was added to 60 ml. of a dry acetone solution containing 6.0 Gm. (0.02 mole) of sodium pentothal. After 1 day at room temperature, the sodium bromide was filtered out and the acetone evaporated. The resulting yellow syrup was dissolved in 30 ml. of benzene and extracted with three 10-ml. portions of 2% sodium hydroxide solution, followed by extraction with water until the base was removed. After drying over anhydrous sodium sulfate, the benzene was evaporated, giving 7.8 Gm. (87%) of a hard yellow amorphous powder. Analytical data shown in Table I and the absence of imino group absorption at 3.1μ in the infrared spectrum indicate the formation of a di-glucoside. Deacetylation was accomplished by the standard sodium methoxide method resulting in a yellow amorphous solid which was very water soluble but noncrystallizable.

SYNTHETIC PROOF OF NITROGEN GLUCOSIDE BOND

It is obvious that the glucoside bonds could be either through the nitrogen or enolic oxygen atoms of the barbiturate. Therefore, the acetylated di-nitrogen glucoside of barbital was synthesized in an unambiguous manner. The physical properties of this compound are identical to those of the glucoside synthesized from acetobromoglucose and barbital, indicating that a di-nitrogen glucoside was actually formed. The synthetic approach which was chosen involves the following steps: (*a*) synthesis of sym-di-(tetra-O-acetyl-β-D-glucopyranosyl)-urea, and (*b*) condensation of the acetylated di-glucose-urea derivative with diethylmalonyldichloride.

(*a*) **Sym-di-(tetra-O-acetyl-β-D-glucopyranosyl)-urea.**—A mixture of urea (0.60 Gm., 0.01 mole), powdered Drierite (10 Gm.), silver oxide (2.32 Gm., 0.01 mole), and acetobromoglucose (8.22 Gm., 0.02 mole) in 10 ml. of dimethylsulfoxide (DMS) was

TABLE I.—BARBITURATE GLYCOSIDES

Compound	Yield, %	M.p., °C.	$[\alpha]_D^{25}$	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
5,5-Diethyl-1,3-di-(tetra-O-acetyl-β-D-glucopyranosyl)-barbituric acid	14.0	173–174	−28.7° (C, 4.17; $CHCl_3$)	$C_{36}H_{48}N_2O_{21}$	...	...	...	...	3.31	3.29
5-Allyl-5-(1-methylbutyl)-1,3-di-(tetra-O-acetyl-β-D-glucopyranosyl)barbituric acid	15.3	77–82	−29.3° (C, 2.49; $CHCl_3$)	$C_{40}H_{54}N_2O_{21}$	53.45	53.30	6.01	6.02	3.12	3.27
5-Ethyl-5-phenyl-1,3-di-(tetra-O-acetyl-β-D-glucopyranosyl)-barbituric acid	7.5	90–96	−8.4° (C, 4.05; $CHCl_3$)	$C_{40}H_{48}N_2O_{21}$	...	...	...	...	3.14	2.93
5-Ethyl-5-isoamyl-1-(tetra-O-acetyl-β-D-glucopyranosyl)-barbituric acid	7.5	47–52	−18.6° (C, 4.24; $CHCl_3$)	$C_{25}H_{36}N_2O_{12}$	54.25	53.35	6.51	6.68	5.06	5.35
5-Ethyl-5-(1-methylbutyl)-1,3-di-(tetra-O-acetyl-β-D-glucopyranosyl)-2-thiobarbituric acid	87.0	62–67	Due to yellow color, optical rotation could not be determined	$C_{39}H_{18}N_2O_{20}S$	52.24	52.57	5.36	5.67	3.12	3.46
5,5-Diethyl-4-(tetra-O-acetyl-β-D-glucopyranosyloxy)-2,6-pyrimidinedione[a]	5.3	178–179	+8.04° (C, 4.17; $CHCl_3$)	$C_{22}H_{30}O_{12}N_2$	51.35	51.20	5.88	5.75	5.45	5.66

[a] Synthesized by procedure of Snyder and Link (11).

TABLE II.—PHARMACOLOGICAL RESULTS

Barbiturate	Anesthetic Dose of Sodium Barbiturate,[a] mg.	Type of Glucoside	Dose of Barbiturate Glucoside,[a] mg.	Results
Barbital	200	Di-nitrogen	1500	Inactive
Barbital	200	Mono-enolic tetraacetate[a]	1200	Inactive
Phenobarbital	100	Di-nitrogen	1000	Inactive
Secobarbital	40	Di-nitrogen	1000	Inactive, painful
Amobarbital	200	Mono-nitrogen	2000	Inactive
Thiopental	40	Di-nitrogen	500	Inactive

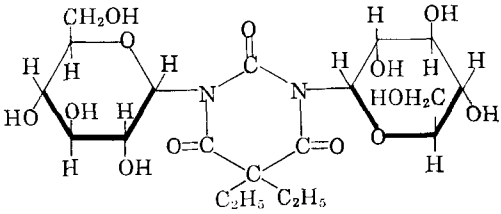
[a] All were intraperitoneal injections of aqueous solutions except the mono-enolic glucoside tetraacetate of barbital which was in the form of a 1% acacia aqueous suspension. The doses are in mg. of compound per Kg. of rat.

stirred for 12 hours (reaction of all acetobromoglucose was indicated by negative test with silver nitrate T.S.). After filtering and washing with dry ether, the DMS was distilled (58°, 5 mm.) resulting in an opaque syrup. It was not possible to crystallize the syrup which was used in the following reaction with diethylmalonyldichloride.

(*b*) **Condensation of Sym-di-(tetra-O-acetyl-β-D-glucopyranosyl)-urea with Diethylmalonyldichloride.**—Diethyldiethylmalonate was prepared from 0.5 mole of diethylmalonate according to the procedure of Vogel (15), but was not isolated. The alcoholic filtrate containing diethyldiethylmalonate was then saponified with 2.5 moles of sodium hydroxide and the alcohol distilled. After extraction with ether and crystallization from chloroform, the yield of diethylmalonic acid was 15.1 Gm. (19%), m.p. 124–125°.

Diethylmalonyldichloride was prepared from 8.0 Gm. of diethylmalonic acid and 20.8 Gm. of phosphorus pentachloride according to standard procedure. The acid chloride was distilled at 85–89° (14 mm.); yield 9.1 Gm. (92%), b.p. 199°.

Diethylmalonyldichloride, 1.37 Gm. (0.007 mole) was added to 50 ml. of an ether solution containing 3.5 Gm. of the syrup obtained in (*a*) and 1.2 Gm. (0.015 mole) of pyridine. After 20 hours reaction time at room temperature, the mixture was filtered and residue washed with dry ether. The ether filtrate was then extracted with two 50-ml. portions of 5% sodium carbonate solution and two 50-ml. portions of water. After drying the ether solution with anhydrous sodium sulfate, the solvent was removed *in vacuo*. Crystallization occurred very slowly after 2 days from alcohol-water, after seeding with the acetylated barbital diglucoside which was obtained by the Bodendorf procedure. The crystals were filtered after 10 days and washed with 10 ml. water. After recrystallization from absolute methanol, the yield of fine needles was 480 mg.; m.p. 174–175°. A mixed melting point with the acetylated barbital diglucoside obtained by direct condensation of acetobromoglucose and barbital showed no depression. This unambiguous synthesis indicated that the structure of the barbital di-nitrogen glucoside is that shown below



PHARMACOLOGICAL TESTING

A comparison was made between the barbiturate glucosides and the corresponding sodium barbiturate salts for hypnotic activity in albino rats. The lack of hypnotic activity of the barbiturate glucosides was demonstrated with intraperitoneal injections of 6 to 25 times the hypnotic dose of the corresponding barbiturate salt. Both the enolic tetraacetate and nitrogen-type glucosides were inactive. The results are summarized in Table II.

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Comparative Activities and Toxicities IV

Dinitro- and Dichloronitro- Phenylamino Acids and Esters

By WILLIAM O. FOYE and DAVID B. MORTENSON

N-2,4-Dinitrophenyl- and N-4,5-dichloro-2-nitrophenyl-α-amino acids and esters have been synthesized in an attempt to remove the toxic effects of the nitro and dichloronitro benzene nucleus to the host without removing antimicrobial activity. The esters were more effective than the free amino acids in reducing acute toxicity to mice. Some antibacterial and antifungal activity was retained in these derivatives, particularly the N-4,5-dichloro-2-nitrophenyl α-amino acids.

BELLENGHI and Wittgens (1) have reviewed the antimycotic and antibacterial effects of nitrovinyl derivatives in the benzene, thiophene, and furan series and shown that the presence of the nitro group in these compounds generally enhanced these activities. Other nitro-containing furans (2), quinolines (3), and phenols (4–6) have been tested for antibacterial action, and the introduction of the nitro group has generally enhanced the effectiveness of the compounds against bacteria. Serious toxic effects may be observed from the use of nitro-containing compounds, however, such as Tien, *et al.* (7), found in a series of N-arylglycines that showed good antitubercular activity *in vitro*.

Previously, in this laboratory, successful use was made of D-glucamine as a detoxifying agent for nitro-containing and halogenated benzene derivatives (8), but with the exception of the 2-nitro-4,5-dichlorophenyl residue, antibacterial activity was also removed by introduction of the glucamine moiety. Introduction of the glycine and glycine ethyl ester residues into the nitro benzenes did not cause such a striking detoxication effect, but neither was the antibacterial spectrum diminished so drastically. Since then, a series of N-2,4-dinitrophenyl and N-2-nitro-4,5-dichlorophenyl derivatives of several representative α-amino acids and their ethyl esters have been prepared, and we now can report the effects these amino acids and esters have on the toxicities and antibacterial spectra of the dinitro and dichloronitro benzene residues. The physical properties of the compounds prepared are listed in Table I.

Received October 20, 1961, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.

Accepted for publication November 29, 1961.

Abstracted from a thesis submitted by D. B. Mortenson in partial fulfillment of the requirements for the degree of Master of Science, 1959.

This project was supported in part by a grant (A-1914) awarded by the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health.

The authors acknowledge with appreciation the cooperation of Drs. K. K. Chen and E. Rohrmann, and W. S. Boniece, M. Clarkson, D. Fleming, and C. L. Rose in carrying out the toxicity and antimicrobial tests and making the results available for publication.

The relative detoxifying effects of the various amino acids and esters may be seen in Table II, where the acute i.p. toxicities in mice are recorded. With the exception of the serine derivative, the N-2,4-dinitrophenyl amino acids were found to be lethal to mice within the dose range of 400–800 mg./Kg. A greater range of toxicities, and generally greater toxicity, was found with the N-4,5-dichloro-2-nitrophenyl amino acids, the glycine and serine derivatives conferring the largest detoxifying effects. The amino acid esters showed a more dramatic detoxifying effect, on the 2-nitro-4,5-dichlorophenyl residue, the lethal doses to mice exceeding 800 mg./Kg. The leucine ester was not obtained in crystalline form for comparison.

Antibacterial screening of the compounds prepared (with the exception of the glycine ester derivative) was carried out by using a small quantity of the dry, powdered material placed on an agar plate. These tests (Table III) showed that in approximately two-thirds of the cases (10 organisms were used) growth inhibition resulted, although the success of this method is dependent upon the ability of the compounds to diffuse through agar. Lack of solubility of the compounds in suitable solvents prevented the use of more customary procedures.

Antimicrobial testing against a larger number of bacteria and several fungal plant pathogens was carried out at the Lilly Research Laboratories with some of the compounds, using the agar dilution technique (see Table IV). N-(2,4-Dinitrophenyl)-methionine, N-(2,4-dinitrophenyl)-leucine, and N-(2,4-dinitrophenyl)-phenylalanine showed no growth inhibition of the organisms tested below a concentration of 200 mcg./ml., but the results obtained with the other compounds tested are shown in Table IV. The 2,4-dinitrophenyl amino acids, the most consistent inhibitors on the agar plate, were found to be without antibacterial activity at the dilutions employed. The 4,5-dichloro-2-nitrophenyl

TABLE I.—N-2,4-DINITROPHENYL- AND N-4,5-DICHLORO-2-NITROPHENYL-DL-AMINO ACIDS AND ESTERS

Compound	M.p., °C.	Purification Solvent	Yield, %	Formula	Analyses, %—Calcd.	Found
N-(2,4-Dinitrophenyl)-glycine	203.5–205.5[a]	Aq. methanol	44	$C_8H_7N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-phenylalanine	217.5–219[b]	Ether-pet. ether	19	$C_{15}H_{13}N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-leucine	134.5–135.5[c]	Ether-pet. ether	32	$C_{12}H_{15}N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-methionine	122–123.5[d]	Ether-pet. ether	17	$C_{11}H_{13}N_3O_6S$	...	...
N-(2,4-Dinitrophenyl)-DL-serine	204–206[e]	Ether-pet. ether	33	$C_9H_9N_3O_7$	...	...
N-(4,5-Dichloro-2-nitrophenyl)-glycine	198–199	Aq. ethanol	31	$C_8H_6Cl_2N_2O_4$	C, 36.30 H, 2.30	36.50 2.30
N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine	193–194	Aq. ethanol	8	$C_{15}H_{12}Cl_2N_2O_4$	C, 50.70 H, 3.40	51.15 3.34
N-(4,5-Dichloro-2-nitrophenyl)-DL-leucine	172.5–173	Aq. ethanol	21	$C_{12}H_{14}Cl_2N_2O_4$	C, 44.88 H, 4.39	45.07 4.48
N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine	135–136.5	Aq. ethanol	26	$C_{11}H_{12}Cl_2N_2O_4S$	C, 38.94 H, 3.56	39.08 3.77
N-(4,5-Dichloro-2-nitrophenyl)-DL-serine	203.5–204	Aq. methanol	39	$C_9H_8Cl_2N_2O_5$	C, 36.63 H, 2.73	36.67 2.96
Ethyl N-(4,5-dichloro-2-nitrophenyl)-glycinate	122–123	Aq. ethanol	24	$C_{10}H_{10}Cl_2N_2O_4$	C, 40.80 H, 3.40	41.07 3.58
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-phenylalaninate	104–106	Aq. ethanol	58	$C_{17}H_{16}Cl_2N_2O_4$	C, 51.90 H, 4.10	52.88 4.22
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-methioninate	67– 69	Abs. ethanol	22	$C_{13}H_{16}Cl_2N_2O_4S$	C, 42.50 H, 4.40	42.01 4.58
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-serinate	127–128	Aq. ethanol	19	$C_{11}H_{12}Cl_2N_2O_5$	C, 40.90 H, 3.70	40.80 3.70

[a] Lit. (12) m.p. 203–204°. [b] Lit. (12) m.p. 204–206°. [c] Lit. (13) m.p. 133°. [d] Lit. (12) m.p. 117–118°. [e] Lit. (12) m.p. 200–202°.

TABLE II.—ACUTE INTRAPERITONEAL TOXICITIES[a] IN MICE

No.	Compound	Dose, mg./Kg.	No. Died/No. Used	Toxic Signs[b]
1	N-(2,4-Dinitrophenyl)-glycine	400	1/5	Died in 6 hr.
		800	5/5	Died in 3 hr.
2	N-(4,5-Dichloro-2-nitrophenyl)-glycine	400	2/5	Died in 6 hr.
		800	5/5	Died, 1–3 hr.
3	N-(4,5-Dichloro-2-nitrophenyl)-glycine ethyl ester	400	0/5	
		800	1/5	Died overnight
4	N-(2,4-Dinitrophenyl)-DL-phenylalanine	400	3/5	Died in 8 hr.
		800	5/5	Died, 10–30 min.
5	N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine	200	2/5	Died in 48 hr.
		400	5/5	Died in 1 hr.
6	N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine ethyl ester	400	1/5	Died in 2 days
		800	1/5	Died in 2 days
7	N-(2,4-Dinitrophenyl)-DL-leucine	400	1/5	Died in 2 days
		800	5/5	Died in 6 hr.
8	N-(4,5-Dichloro-2-nitrophenyl)-DL-leucine	200	1/5	Died in 3 days
		400	5/5	Died in 8 hr.
9	N-(2,4-Dinitrophenyl)-DL-methionine	400	1/5	Died in 6 hr.
		800	5/5	Died in 3 hr.
10	N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine	100	3/5	Died overnight
		200	4/5	Died in 6 hr.
11	N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine ethyl ester	400	0/5	
		800	0/5	Some ataxia
12	N-(2,4-Dinitrophenyl)-DL-serine	400	0/5	
		800	1/5	Died overnight
13	N-(4,5-Dichloro-2-nitrophenyl)-DL-serine	400	1/5	Died overnight
		800	5/5	Died, 3–4 hr.
14	N-(4,5-Dichloro-2-nitrophenyl)-DL-serine ethyl ester	400	0/5	
		800	0/5	None

[a] Determined at the Lilly Research Laboratories by C. L. Rose. [b] Observations were made for 1 week after single injections of compound.

amino acids in general showed some antibacterial activity, although none approached the breadth of spectrum previously found for N-(4,5-dichloro-2-nitrophenyl)-D-glucamine (8). Only two of the esters were tested, the glycine and phenylalanine derivatives. The former showed no activity while the latter showed some activity, particularly against the fungal plant pathogens. N-(2,4-Dinitrophenyl)-glycine was also tested in mice against two types of virus, *Streptococcus pyogenes*, *Proteus vulgaris*, and typhoid organisms, but was found ineffective.

TABLE III.—ANTIBACTERIAL SCREENING[a]

Test Organism	Compound Number 1	2	4	5	6	7	8	9	10	11	12	13	14
Staphylococcus aureus	−	−	−	−	+	−	−	−	−	+	−	−	+
Sarcina lutea	−	−	−	−	−	−	−	−	−	−	−	−	+
Bacillus subtilis	−	+	+	+	+	−	+	−	+	+	−	−	+
Escherichia coli	−	+	−	+	+	−	−	−	−	+	−	−	+
Aerobacter aerogenes	−	+	+	+	+	−	+	−	+	+	−	−	+
Serratia marcescens	−	+	+	+	+	−	+	−	+	+	−	−	+
Alcaligenes faecalis	−	−	−	−	+	−	−	−	−	−	−	−	+
Klebsiella pneumoniae	−	−	−	−	+	−	−	−	−	−	−	−	+
Proteus vulgaris	−	−	−	−	−	−	−	−	−	−	−	−	+
Pseudomonas aeruginosa	−	−	−	+	+	−	+	−	+	+	−	+	+

[a] A negative sign denotes inhibition of growth, which was usually over a radius of 2–3 mm. on the agar plate. A small quantity of the dry, powdered compound was placed on the plate.

TABLE IV.—ANTIBIOTIC SPECTRA[a]

Test Organism	Inhibitory Concentration, mcg./ml.[b] Compd. No. 5	6	8	10	13
Staphylococcus aureus	200	...	50	100	..
Staphylococcus albus	...	...	50	100	..
Bacillus subtilis	200	...	50	100	..
Sarcina lutea	200	...	50	100	..
Mycobacterium tuberculosis	200	200	50	50	50
Mycobacterium avium	...	...	100	100	..
Escherichia coli	...	...	...	...	..
Proteus vulgaris	...	...	...	100	..
Pseudomonas aeruginosa	...	...	...	[c]	..
Aerobacter aerogenes	...	...	...	...	..
Klebsiella pneumoniae	...	...	...	...	..
Salmonella enteritidis	...	...	...	...	..
Shigella paradysenteriae	...	...	...	...	..
Saccharomyces pastorianus	...	...	...	...	..
Candida albicans	...	...	...	...	..
Trichophyton rubrum	...	200	...	...	..
Trichophyton interdigitale	...	200	100	...	..
Brucella bronchiseptica	...	...	...	5	..
Vibrio metschnikovii	...	...	100	...	..
Alternaria solani	200	...	...	...	..
Aspergillus niger	...	200	...	...	..
Ceratostomella ulmi	...	200	...	...	..
Colletotrichum lagenarium	...	...	...	...	..
Colletotrichum phomoides	...	200	...	...	..
Colletotrichum pisi	...	200	...	...	..
Endoconidiophora fagacearum	...	200	...	...	..
Sclerotinia fructicola	...	200	...	...	..

[a] Carried out at the Lilly Research Laboratories by M. Clarkson and D. Fleming. [b] The agar dilution technique was used, the bacteria being observed for 48 hours and the fungal plant pathogens for 72 hours. [c] Test organism did not grow sufficiently well to establish an end point; where no inhibitory concentration is reported, the organism was not inhibited by 200 mcg./ml. of compound.

It may be concluded from these comparisons that the introduction of the α-amino acid residue into the *m*-dinitrobenzene molecule shows a small but definite detoxifying effect (*m*-dinitrobenzene killed four of five mice used at 400 mg./Kg., and 2,4-dinitroaniline killed all of five mice at 400 mg./Kg.). The amino acid esters, however, showed a greater ability to detoxify, when attached to the 4,5-dichloro-2-nitrophenyl nucleus. Again, as in our previous study (8), loss of toxicity to mice generally paralleled loss of toxicity to microorganisms, although use of the amino acid ester derivatives holds some promise that a selective detoxifying effect without corresponding loss of antimicrobial ability may be achieved.

EXPERIMENTAL

The melting points were taken on a Fisher-Johns block and are uncorrected. Analyses were done either by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass.

N-2,4-Dinitrophenylamino Acids.—These compounds were prepared either by the method of Abderhalden and Blumberg (9) or Sanger (10). A representative synthesis follows. DL-Phenylalanine (2.0 Gm., 0.012 mole) and sodium bicarbonate (4.0 Gm., 0.048 mole) were dissolved in 50 ml. of water, and 2.5 ml. (0.220 mole) of 2,4-dinitrofluorobenzene in 100 ml. of ethanol was added. The mixture was shaken at room temperature for 2 hours, and the ethanol was then removed by evaporation. The resulting mass was dissolved in water, shaken three times with ether, and the aqueous solution was acidified. The yellow precipitate was collected, washed with ice water, and dissolved in acetone. The acetone solution was shaken with anhydrous sodium sulfate, filtered, and evaporated to a small volume. An equal volume of benzene was added, followed by petroleum ether until the product precipitated. The compound was dried in air, dissolved in ether, and reprecipitated with petroleum ether. This precipitation procedure was repeated several times until well-formed crystals were obtained. The yield was 0.78 Gm. (19%) of yellow crystalline compound, m.p. 217.5–219° [lit. (10) m.p. 204–206°].

N-4,5-Dichloro-2-nitrophenylamino Acids.—A typical procedure follows. Glycine (0.75 Gm., 0.01 mole) and sodium bicarbonate (1.68 Gm., 0.20 mole) were dissolved in 50 ml. of warm water and 50 ml. of warm ethanol was added. 1,2-Dinitro-4,5-dichlorobenzene (2.37 Gm., 0.01 mole) [prepared by the procedure of Hartley and Cohen (11)] was dissolved in 100 ml. of warm aqueous ethanol (50:50). The two solutions were mixed and boiled until no odor of ethanol could be detected. The solution was cooled and filtered, and the residue was dissolved in warm water. The aqueous solution was acidified,

and the yellow product was filtered and recrystallized from aqueous ethanol. The yield was 0.83 Gm. (31%); m.p. 198–199°.

N-4,5-Dichloro-2-nitrophenylamino Acid Ethyl Esters.—The following procedure is representative. N-4,5-Dichloro-2-nitrophenyl-DL-serine (3.0 Gm., 0.008 mole) was dissolved in 100 ml. of absolute ethanol. The solution was heated to 70°, and dry hydrogen chloride was introduced, with stirring, for a period of 2 hours. The resulting solution was evaporated to a small volume and cooled. Water was added until the ester precipitated, and the precipitate was collected. This precipitation from ethanol was repeated, and the product was dried over calcium chloride. The yield was 0.60 Gm. (19%); m.p. 127–128°.

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Phytochemical Investigation of *Amphipterygium adstringens*

By EDWARD E. GONZALEZ† and JAIME N. DELGADO

A general phytochemical investigation was conducted on *Amphipterygium adstringens*, Schlecht. Numerous qualitative studies were conducted and several conventional techniques were employed in the detection, isolation, purification, and chemical characterization of constituents. Qualitative chemical tests showed the presence of phytosterols, glycosides, and tannins, and the absence of alkaloids and phloroglucides. An organic acid and a steroidal saponin were isolated from the diethyl ether and *n*-butanol extracts, respectively. The *p*-nitrobenzyl and *p*-phenylphenacyl derivatives of the carboxylic acid were prepared. The neutralization equivalent and infrared spectrum of the isolated acid were established. Chromatographic and infrared studies of the saponin have been reported.

THIS PHYTOCHEMICAL study was initiated primarily because preliminary screening tests indicated that *Amphipterygium adstringens*, Schlecht (Fam. *Julianiaceae*) possessed a certain degree of anticancer activity (1). This observation, together with a survey of the available literature which revealed no apparent report on the exact chemistry of this botanical species, stimulated interest and prompted this investigation. Furthermore, since Martinez (2) briefly makes note of *Amphipterygium adstringens* in the second part of his book which deals only with medicinal plants lacking scientific investigation, it was felt that the project was worthy of exploration.

A survey of botanical literature revealed disagreements in the scientific nomenclature of *Amphipterygium adstringens*, Schlecht. Some of the early reports refer to the species as *Hypopterygium adstringens*. In the more recent literature the generic name is given as *Juliania*, family *Julianiaceae*. *Amphipterygium adstringens*, commonly known as "Cuachalalate," is a small tree indigenous to Mexico, growing from Michoacán to Morelos and Puebla to Oaxaca. The assumed medicinal effects of the bark are briefly described by Martinez (2). Concoctions of the bark are used extensively for alleviating numerous conditions, including malaria, intermittent fever, ulcers, and cancer of the gastrointestinal tract.

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Accepted for publication November 30, 1961.
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EXPERIMENTAL

Source and Preparation of the Plant Material.—The chemical studies were conducted on authenticated samples.[1] The plant material used in this study consisted of the outer, dried bark of *Amphipterygium adstringens*, free of adulteration and adhering foreign matter. The hard, bulky pieces of bark were first reduced in size with a Wiley grinding laboratory mill, and then pulverized to a No. 20 mesh size in a W. J. Fitzpatrick, model D, comminuting machine. The ground material was then stored in tight metal containers to avoid contamination and possible deterioration.

Extraction Studies.—Suitable, accurately weighed samples of powdered material were extracted successively with a series of solvents in a Soxhlet continuous extraction apparatus by the Rosenthaler method (3). The solvents were used in the order of increasing polarity, and the extraction with a given solvent was continued until an aliquot of the colorless percolate left no residue when evaporated to dryness. The percentage of extractives were as follows: petroleum ether (b.p. range 30–60°), 6.11%; anhydrous ether, 4.62%; chloroform U.S.P., 0.82%; anhydrous ethanol, 9.61%; 70% ethanol, 4.07%.

Phytosterols.—In an attempt to determine the presence of phytosterols, effective application was made of the digitonin reaction according to the method described by Fieser and Fieser (4). The nonsaponifiable fraction of the petroleum ether[2] extract gave a positive reaction with digitonin, forming a white crystalline substance which melted at 139–143°. In order to substantiate fully the results of the digitonin reaction, other more general and classical qualitative tests were applied to fractions of the nonsaponifiable material. Positive results were obtained with Hesse's, Moleschott's, Liebermann's, and Hirschsohn's reactions.

Phloroglucides.—The Coward-Harris (5) procedure was employed as the basis for the qualitative analysis for phloroglucides. No precipitation was observed indicating their absence.

Alkaloids.—The chloroform extract of the whole drug (200 Gm.) was examined for alkaloids by the proximate assay method for alkaloids (6). Since the resulting extract gave a negative test with Mayer's reagent but produced slight opalescence with gold chloride T.S. and tannic acid T.S., an attempt to isolate alkaloids was made on a larger portion of crude drug following the procedure of Kleber and Gisvold (7). Negative results were obtained with Mayer's reagent, Lugol's solution, gold chloride T.S., picric acid T.S., and phosphomolybdic acid T.S., conclusively indicating the absence of alkaloidal substances in the plant material.

Glycosides.—A modified procedure of the Stas-Otto method was employed in the qualitative determination for glycosides. A mixture of plant material (300 Gm.) and ethanol containing tartaric acid (2%) was refluxed for approximately 12 hours. After the solvent was recovered by filtration, the marc was washed several times with hot ethanol and then pressed as free as possible of solvent. These filtrates were combined and evaporated to dryness in a Rinco rotatory evaporator under reduced pressure using steam as the source of heat. The residue was washed with hot distilled water (four 50-ml. portions) and the aqueous solution was extracted with diethyl ether (four 50-ml. portions) in a separator. The combined ethereal extracts were evaporated to dryness and the residue dissolved in ethanol. Aliquots of this solution were tested with Molisch reagent. Positive results were obtained, indicating the presence of carbohydrate substances and/or glycosides.

Tannins.—A hydroalcoholic (50% ethanol) extract of the plant material was prepared and aliquots of the solution were checked for tannins with various test solutions. A positive reaction was obtained with potassium dichromate, gelatin, ferric chloride, and uranyl acetate reagents. The tannins were isolated by the Feist and Bestehorn method (3) and an attempt was made to classify the tannins according to their specific chemical reactions with various reagents (8). In order to prevent one intense color from obscuring or masking another, several tannin solutions of ranging concentration were prepared. Positive results were obtained with fresh bromine water and ferric chloride T.S., while negative results were obtained with vanillin-hydrochloride and ammonium molybdate.

The production of a green color with ferric chloride T.S. indicated a catechol nucleus (two adjacent free phenolic groups) in the molecule, rather than a pyrogallol nucleus (three adjacent free phenolic) groups). The fact that the tannins were precipitated with bromine water reveals that they are of the nonhydrolyzable type since the hydrolyzable tannins do not react in this manner. Negative results obtained with ammonium molybdate and vanillin-hydrochloride indicate the absence of gallic acid and phloroglucin tannins, respectively.

Examination of Diethyl Ether Fraction.—The marc which had been previously exhausted with petroleum ether was extracted with diethyl ether by cold percolation. The ethereal extract was concentrated under reduced pressure to 50% of its original volume and placed in the deep freezer at −10° for several days. A substantial amount of pyramidal shaped crystals precipitated from the ethereal solution. The yellowish crystals which formed were removed by filtration and washed several times with cold acetone and methanol. These washings removed most of the colored impurities. The crude crystalline substance was recrystallized from ethanol and dried, *in vacuo*, at 80° with potassium hydroxide in an Abderhalden drying pistol. The crystalline material melted at 165–166° and was acidic to both litmus paper and phenolphthalein T.S. The crystals proved to be insoluble in water, methanol, acetone, concentrated hydrochloric acid, and sulfuric acid; sparingly soluble in hot methanol, cold ethanol, chloroform, and carbon tetrachloride. On the other hand, the substance exhibited a high degree of solubility in hot ethanol, sodium hydroxide solution, alcoholic potassium hydroxide, and sodium bicarbonate solution. Some of the qualitative tests that were applied for the classification and partial chemical characterization are summarized as follows: (*a*) ferric chloride test for phenols, negative; (*b*) Molisch test for carbohydrates, negative; (*c*) ceric ammonium nitrate test for alcohols, negative; (*d*)

[1] Kindly furnished by Dr. H. R. Schmidt, research pharmacognosist, Department of Research, S. B. Penick and Co., New York, N. Y.

[2] Unless otherwise specified, petroleum ether refers to technical grade petroleum benzin, boiling range 30–60°.

Liebermann-Burchard test for sterols, negative; (*e*) Fuchsin-aldehyde test, negative; (*f*) lead acetate test, positive; (*g*) picric acid test, negative; (*h*) cupric sulfate test, positive; (*i*) iodine test, negative; (*j*) potassium permanganate test, positive (ppt.); (*k*) sodium fusion test for nitrogen, phosphorus, and sulfur, negative. The preceding data eliminated several possibilities and indicated that the isolated substance was possibly an organic acid.

Anal.—Found: C, 78.26; H, 10.99. The neutralization equivalent of the acidic crystalline substance was determined according to the standard analytical method described by Niederl and Niederl (9). The average neutralization equivalent was determined to be 550.20.

Esterification.—The *p*-nitrobenzyl and *p*-phenylphenacyl derivatives of the organic acid were prepared from the corresponding bromides according to the procedures of Kelly (10) and Drake (11), respectively. These two particular derivatives were chosen because there is usually a considerable difference in the melting point of the *p*-nitrobenzyl and *p*-phenylphenacyl ester of any one acid. During the esterification process, it was essential to maintain the reaction mixture slightly acidic since alkalies could hydrolyze the parent halides to the corresponding alcohols. The prepared *p*-nitrobenzyl ester melted at 72–73° and the *p*-phenylphenacyl ester at 127–128°.

The neutralization equivalent value and the melting points of the prepared derivatives did not compare with any values reported in the literature.

Infrared Analysis.—A potassium bromide wafer of the crystalline substance was prepared for the infrared spectrum analysis since it was not sufficiently soluble in chloroform. The infrared spectrum was determined in an infrared recording spectrophotometer, model 4-55, Baird Associates, Inc. The absorption spectrum of the isolated acid is shown in Fig. 1.

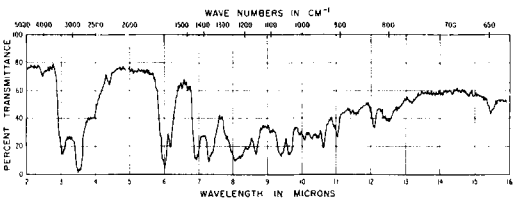


Fig. 1.—Infrared spectrum of the unknown acid in potassium bromide wafer.

The broad absorption bands between 3.0 and 3.6 μ (3310–2860 cm.$^{-1}$) fall within a frequency range which is normally assigned to carboxylic acids; however, the specific band observed under 3.5 μ (2850–2860 cm.$^{-1}$) may be conferred also by —CH_2— vibrations (12). Inspection of the spectrum for the carboxyl function disclosed a stretching band with a shoulder near 6.0 μ (1700–1650 cm.$^{-1}$). The absorption band at 6.9 μ (1465–1400 cm.$^{-1}$) could be manifested by the covalent carbon-oxygen bond of the carboxyl group. Some indications of the presence of an isopropyl group is found in the absorption band observed in the 7.3–7.4 μ (1370–1350 cm.$^{-1}$) region (12). Furthermore, the bands appearing near 8.1 μ (1250 cm.$^{-1}$) and 8.7 μ (1150 cm.$^{-1}$) give reason to suspect its presence. The other weak peaks noted above 9.0 μ may possibly be deformation frequencies associated with either propyl or carboxyl group vibrations.

Examination of Aqueous Fraction.—During the preliminary chemical studies, it was noted that the aqueous extract produced vigorous foaming when agitated. This observation, and the fact that the Stas-Otto test for glycosides was positive, suggested the possibility of existing saponins. In order to provide more convincing evidence, the aqueous extract of the plant material was extracted with hot *n*-butanol by means of a separator and the residue obtained after evaporation of the butanol was tested with antimony trichloride T.S. (13). A positive reaction was observed with the production of a yellow color.

In order to isolate an adequate amount of saponin, 10 Kg. of powdered bark were extracted by means of cold percolation in a large metal percolator (36 in. high and 14 in. inside diameter). The percolation was continued at a moderate rate over a period of several days until 15 gallons of liquid were collected. Extreme care was exercised to avoid fermentation during the long period of slow maceration and percolation. The aqueous percolate was lyophilized to dryness in a F. J. Stokes Lyophilyzer, model 2004L X 3, and the combined residues washed and refluxed in *n*-butanol.[3]

After filtration, the *n*-butanol extract was concentrated by distillation, *in vacuo*, at 55–60° to a small volume. Cold anhydrous acetone (200 ml.) was added to the contents of the flask causing the precipitation of a light amorphous substance. A total of 13.4 Gm. of crude saponin was recovered by the aforementioned process. The solid amorphous substance was hygroscopic and soluble in water, with considerable foaming and opalescence. It was soluble in methanol and ethanol, insoluble in petroleum ether, diethyl ether, chloroform, and acetone. Some of the basic qualitative tests that were applied for the chemical classification are summarized as follows: (*a*) Molisch test, positive; (*b*) ferric chloride test, negative; (*c*) Liebermann-Burchard test, negative; (*d*) Seliwanoff test, negative; (*e*) antimony trichloride test, positive; (*f*) basic lead acetate test, positive; (*g*) the addition of ether to an aqueous solution caused the formation of a gelatinous precipitate; (*h*) the addition of sulfuric acid (1 ml.) to a concentrated aqueous solution produced a crimson color. The preceding chemical data substantiated that the amorphous substance under consideration was a saponin.

Hydrolysis of Saponin.—Hydrolysis of the above described glycosidal compound was accomplished by means of strong mineral acid in alcohol and water mixtures. A saponin sample (4 Gm.) was dissolved in a hydroalcoholic mixture composed of distilled water (50 ml.) and ethanol (25 ml.), to which was added concentrated sulfuric acid (8.6 ml.). The acidic mixture was refluxed vigorously on a steam bath for 6.5 hours. The foaming that occurred on heating was controlled by the addition of *n*-amyl alcohol (5 ml.). The reaction mixture was allowed to cool and the contents poured into a separator and extracted with warm chloroform (three 50-ml. portions). The combined chloroform extracts were con-

[3] Grateful acknowledgment is made to Dr. F. P. Cosgrove for his technical assistance in this phase of our study.

TABLE I.—SOLVENT SYSTEMS AND R_f VALUES FOR UNKNOWN AND REFERENCE SAPOGENINS[a]

Solvent System	a	b	c	d	e
Petroleum ether, ml.	400	400	200	100	0
Toluene, ml.	0	50	100	200	400
Ethanol, ml.	10	10	10	30	10
Water, ml.	90	90	90	70	90
Chlorogenin	...	...	...	0.310	0.290
Gitogenin	...	...	...	0.522	0.496
Hecogenin	...	0.227	0.430	0.851	0.878
Tigogenin	0.396	0.641	0.778	0.911	0.960
Diosgenin	0.433	0.645	0.832	0.956	0.988
Sarsasapogenin	0.701	0.862	0.755	1.000	1.000
Smilagenin	0.728	0.859	0.931	1.000	1.000
Unknown genin	0.698	0.865	0.750	1.000	1.000
Unknown genin plus sarsasapogenin	0.695	0.864	0.755	1.000	1.000

[a] Chemically pure (A.R.) reagents were employed in preparing all solvent systems used for chromatographic studies.

centrated to a small volume under reduced pressure in a Rinco rotatory evaporator; the sapogenin was then precipitated by the addition of methanol A.C.S. (10 ml.). The crude genin was recrystallized from methanol and used for chromatographic and infrared studies.

Paper Chromatographic Analysis of Sapogenins.—The apparatus and technique used for the chromatographic analysis of the sapogenins was essentially that described by Heftmann and Hayden (14). Whatman No. 1 filter paper was employed throughout the chromatographic studies and all chromatographic work was carried out in an air-conditioned room at 23°. After resolution was complete, the sapogenin chromatograms were sprayed with a freshly prepared solution of trichloroacetic acid in chloroform (25%) in the manner of Svendsen and Jensen (15). The sheets were heated in a chromatography oven at 90–100° for approximately 15 minutes and, thereafter, the sapogenins were detected by their varying fluorescence under ultraviolet light.

The R_f values of the unknown genin and reference compounds which were chromatographed individually and as mixtures with themselves and with the unknown sample are listed in Table I.

Reference Sapogenins.—Pure authenticated sapogenin samples for experimental study were kindly supplied by Dr. O. Mancera, Syntex Laboratories, Mexico City; and Dr. E. Heftmann, National Institutes of Health, Bethesda, Md. Comparative chromatographic and infrared studies of the unknown genin and reference sapogenins were made. All of the sapogenins were used in a concentration of 1 mg. per ml. of chloroform U.S.P.

Identification of Sugar Moieties of the Saponin.—After removal of the aglycon from the hydrolyzed mixture, the filtrate was neutralized with barium carbonate and filtered. The filtrate was concentrated to a syrupy consistency, dissolved in ethanol, and filtered to give a yellow solution. All of the solutions that were prepared were stored in the refrigerator and warmed to room temperature before using for chromatographic analysis.

Two different solvent systems were employed for the chromatographic resolution of the carbohydrates. One solvent system (S-1) was composed of *n*-butanol (4 parts), distilled water (5 parts), and glacial acetic acid (1 part) (16). The other solvent system (S-2) consisted of 2,6-lutidine (13 parts), and distilled water (7 parts) (17). Because solvent system S-1 was subject to esterification, it was always freshly prepared and used only once. Solvent system S-2 never was used more than twice in the experiments.

An ascending chromatographic technique was used for the separation and identification of the sugar residues. The chromatograms were developed with the mobile phase for approximately 16 hours. After resolution was complete, the dried chromatograms were sprayed with a solution of aniline acid phthalate in water-saturated *n*-butanol and dried for 5 minutes in a chromatography oven at 100°. On sheets developed with this reagent, aldopentoses appeared as red spots and hexoses as green to brown spots. Extreme care was exercised to prevent any destruction of the paper when heating.

Several known sugars of analytical purity were chromatographed individually, and as mixtures with themselves and with the unknown sample. The R_f values obtained with the two different solvent systems are given in Table II.

TABLE II.—SUMMARY OF R_f VALUES FOR CARBOHYDRATES

Compound	R_f Values S-1	R_f Values S-2
D-Xylose	0.353	0.554
D-Glucose	0.295	0.534
D-Galactose	0.306	0.496
D-Rhamnose	0.526	0.641
Unknown sugar mixture	0.297 0.523	0.536 0.644

Infrared Spectra as Proof of Identity.—Since the unknown genin behaved chromatographically like sarsasapogenin, application of infrared spectra was made as a further check in establishing its identity. Chloroform solutions (30%) of the unknown genin and sarsasapogenin were prepared for infrared analysis. The infrared absorption spectrum of each sample was determined in an infrared Beckman spectrophotometer, model IR-5, using 0.059 mm. sodium chloride cells. Chloroform U.S.P. was used as the standard reference sample. The infrared absorption spectra of the isolated sapogenin and sarsasapogenin are shown in Figs. 2 and 3, respectively.

It will be noted that the infrared spectrum of the unknown sapogenin bears great resemblance to that of sarsasapogenin. Each of the two steroidal compounds produced curves with distinctive bands in the

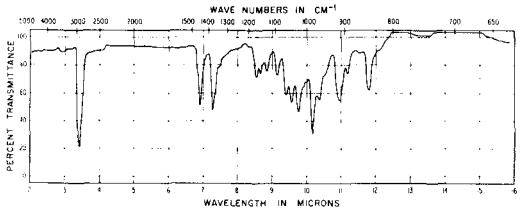


Fig. 2.—Infrared spectrum of the unknown sapogenin in chloroform U.S.P.

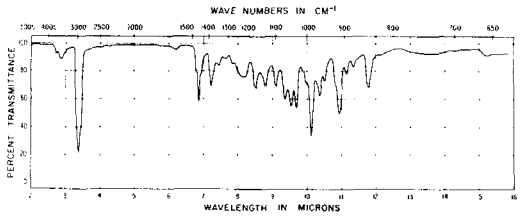


Fig. 3.—Infrared spectrum of sarsasapogenin in chloroform U.S.P.

"finger-print region" which is characteristic of sapogenins. Between 850 and 1350 cm.$^{-1}$ intense absorption bands conferred by the spiroketal side chain are observed (18). The bands of maximum absorption in this highly specific region are located near 987 and 908 cm.$^{-1}$. Those absorption bands observed in the region between 1350 and 1500 cm.$^{-1}$ seem to be associated with vibrations localized in the methyl groups (19). The notable peak located near 2940 cm.$^{-1}$ could be assigned to the —CH_2— vibrations of the molecule. Inspection of the spectra for the 3-hydroxyl group disclosed two bands of medium intensity between 1000 and 1050 cm.$^{-1}$ (20). The absorption bands below 850 and above 1800 cm.$^{-1}$ have not been extensively studied since these regions do not show any prominent features of reasonable consistency.

SUMMARY

A general phytochemical investigation was conducted on *Amphipterygium adstringens*, primarily on the basis of its promising anticancer activity. Numerous chemical qualitative studies were conducted and several conventional techniques were employed in the detection, isolation, purification, and characterization of constituents.

The results of this phytochemical study may be summarized as follows:

1. The qualitative tests for the presence of phytosterols were positive.

2. The determinations for the presence of phloroglucides and alkaloids were negative.

3. The tannins were found to be members of the nonhydrolyzable group containing a catechol type of nucleus.

4. A crystalline substance (m.p. 165–166°) was isolated from the diethyl ether extract of the marc previously exhausted with petroleum ether.

5. On the basis of chemical qualitative tests, solubility properties, and infrared studies, the crystalline substance was characterized as an organic acid. The prepared *p*-nitrobenzyl ester melted at 72–73°, and the *p*-phenylphenacyl ester at 127–128°.

6. The Stas-Otto analysis for the presence of glycosides was positive.

7. A steroidal saponin was isolated from the *n*-butanol extract of the aqueous fraction.

8. Paper chromatographic and infrared analysis of the isolated sapogenin showed that its physical-chemical properties were similar and in direct agreement with those of sarsasapogenin.

9. Hydrolysis of the saponin and subsequent paper chromatographic analysis of the carbohydrate residues showed the presence of D-glucose and D-rhamnose.

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Some Antiarrhythmic Actions of Primaquine, Amodiaquin, and Quinidine

By E. L. HOLLAND and R. S. McCUTCHEON

This study concerns (*a*) the antiarrhythmic properties of amodiaquin and primaquine in comparison with those of quinidine and (*b*) the ionic fluxes caused by acetylcholine and the depression of these fluxes by the antiarrhythmic drugs used. Under (*a*) it was found that both amodiaquin and primaquine are as effective as quinidine in halting atrial fibrillation induced by the injection of acetylcholine after pretreatment with neostigmine. Under (*b*) no ion fluxes were demonstrated after the injection of acetylcholine under the conditions of the experiment.

QUINIDINE is an established drug in the treatment of cardiac disorders. It is the dextroisomer of quinine and is the most active of the cinchona alkaloids in antiarrhythmic activity (1). Quinidine has been reported by Lewis (2) to have the following effects on heart muscle: it depresses excitability, slows conduction, slows the rate, increases the refractory period, and causes a decrease in vagal tone. It has no effect on the contractility of the heart in normal doses.

A search of the literature revealed two synthetic antimalarials that had been studied only a very little for their antiarrhythmic activity. In the few studies that have been done on these drugs it was shown that they are more effective than quinidine in halting atrial fibrillation; these two drugs are amodiaquin and primaquine (3–5).

Arora, *et al.* (3), have shown that amodiaquin is more effective than quinidine in reversing aconitine-induced auricular fibrillation in cats. In another report, Arora, *et al.* (4), have shown that amodiaquin possesses a quinidine-like action on the refractory period of isolated rabbit atria and has a stronger antifibrillatory action on acetylcholine-induced atrial fibrillation in dogs than quinidine. Arora (4) has also reported that amodiaquin differs from quinidine in not protecting against epinephrine-induced ventricular arrhythmias and causing a greater slowing of conduction rate and less prolongation of the refractory period.

Primaquine has been reported by Arora, *et al.* (5), to be more effective than quinidine in protecting dogs against atrial fibrillation induced by the topical application of aconitine and atrial flutter caused by crush stimulation of the atria, but did not show protective action against epinephrine-hydrocarbon-induced ventricular arrhythmias. It has been shown (5) by the electrocardiograph that primaquine increases the refractory period and conduction time of the heart more than did quinidine.

Several authors have also shown that acetylcholine stimulates the efflux of potassium and the influx of sodium in myocardial tissue (6–12). It has also been reported that quinidine depresses this ionic flux (8–10).

EXPERIMENTAL

Antiarrhythmic Study.—One object of these studies was to compare the action of amodiaquin and primaquine with quinidine as to relative effectiveness in halting atrial fibrillation induced by the injection of acetylcholine after the animal had been pretreated with neostigmine.

Five mongrel dogs weighing from 15 to 24 Kg. were used in these studies. Each animal was anesthetized with pentobarbital sodium solution, 35 mg./Kg. intraperitoneally. The anesthetic was supplemented, if necessary, by pentobarbital sodium solution intravenously.

Control electrocardiograms were taken on all animals to determine if any cardiac abnormalities were present. If the animal exhibited any it was rejected.

All electrocardiograms were taken on a Sanborn model 150 four-channel recorder and monitored visually at all times with a Sanborn Visoscope oscilloscope. Standard limb lead II was used and the lead wires were connected to the animal by means of subcutaneous electrode needles.

All injections were made through an indwelling 21-gauge needle placed in either the right or left saphenous vein. To keep the needle from plugging, a very slow (3 to 5 drops/minute) infusion of isotonic sodium chloride was allowed to infuse. The animals were placed in the prone position with the posterior elevated to facilitate the drainage of the copious salivary secretions caused by the acetylcholine.

A method of initiating atrial fibrillation of sufficient duration and predictability to determine the effectiveness of the three drugs was adapted from the following. Leveque (13) produced atrial fibrillation averaging about 25 seconds in dogs by thyroid administration and acetylcholine injection. Loomis and Krop (14) reported that atrial fibrillation could be induced in dogs by the injection of acetylcholine

Received September 13, 1961, from the Oregon State University, School of Pharmacy, Corvallis.
Accepted for publication October 18, 1961.
Submitted by E. L. Holland in partial fulfillment of the requirements for the M.S. degree at Oregon State University.
This research was supported by the Smith, Kline & French Foundation.
Presented to Section Np, American Association for the Advancement of Science, Denver meeting, December 1961.

after the animal had been pretreated with an anticholinesterase agent.

The latter method was found to be the most reliable for inducing atrial fibrillation of extended duration. The cholinesterase agent chosen was neostigmine bromide which was given in a dose of 0.05 mg./Kg. A dose of 0.15 mg./Kg. of acetylcholine 30 minutes after neostigmine was found to produce an atrial fibrillation of long duration with nonfatal respiratory embarrassment.

To summarize, the atrial fibrillation was induced four times by the injection of acetylcholine, after pretreatment with prostigmine, for each treatment with each drug. The average time of the first and second fibrillation served as the control time, and the average time of the third and fourth fibrillation served as the treatment time. Each animal was used three times at random intervals and was treated with each of the antiarrhythmic drugs, fibrillation being produced 12 different times in each.

Drugs and dosages used in the antiarrhythmic study were as follows

Drug[1]	Dose, mg./Kg.
Neostigmine bromide	0.05
Acetylcholine chloride	0.15
Quinidine gluconate	5.00
Amodiaquin hydrochloride	4.00
Primaquine dihydrochloride	4.00

Ion Studies.—The purpose of these studies was to determine the extent to which acetylcholine causes a sodium and potassium flux and to determine whether the three antiarrhythmic drugs studied would depress it.

Serum sodium and potassium were determined using a Beckman model DU spectrophotometer with a flame attachment according to the method of Hawk, *et al.* (15).

All blood samples were drawn from either the right or left saphenous vein or the right or left radial vein in the following order: control (no drug administered except the anesthetic), 20 minutes after prostigmine, immediately after acetylcholine, and shortly after the antiarrhythmic drug. The 5-ml. samples of blood were allowed to coagulate in a refrigerator and then were centrifuged and the clear supernatant serum was drawn off. The serum was diluted 1:250 for the determination of sodium and 1:100 for the determination of potassium. This method is described in Hawk, *et al.* (15).

In this series each dog was used three times as in the antiarrhythmic studies. Blood samples were taken in the order indicated each time the dog was used in the antiarrhythmic study and analyzed for sodium and potassium.

RESULTS AND DISCUSSION

Antiarrhythmic Study.—Each figure of Table I expresses time as minutes:seconds, and is the average of two determinations, showing the average duration of an atrial fibrillation.

The columns labeled "before" show the duration of control fibrillations and the columns labeled "after" are the fibrillatory periods after treatment with the antiarrhythmic drug.

[1] These drugs were kindly furnished by the respective manufacturers: neostigmine bromide, Roche Laboratories; acetylcholine chloride, Merck & Co.; quinidine gluconate, Eli Lilly and Co.; amodiaquin HCl, Parke Davis; primaquine diHCl, Winthrop Laboratories.

It can be seen from Table I that all of the drugs are effective in shortening the duration of the acetylcholine-induced atrial fibrillation. To determine if the three drugs are equally effective, a statistical analysis of the results was done. This is a randomized block experiment with three treatments (drugs) and five replications (dogs).

The difference between the control (before) times and the treatment (after) times was used in the statistical analysis. These differences appear in Table II, the differences being expressed as seconds.

The statistical analysis shows that the three drugs are effective in shortening the duration of the acetylcholine-induced atrial fibrillation, $F = 86.19$.

It also indicates that the dogs are not a variable in this experiment, $F = 0.556$.

And finally the statistical analysis shows that the three drugs in the doses used are equally effective in shortening the duration of the acetylcholine-induced atrial fibrillation. These results do not support those of Arora, *et al.* (3–5), who state that both amodiaquin and primaquine at a dosage level of 4 mg./Kg. are more effective than quinidine at a dosage level of 5 mg./Kg. Our work shows an equal effect of the three drugs at a dosage of 4 mg./Kg. for amodiaquin and primaquine compared to 5 mg./Kg. for quinidine, thus indicating a stronger effect for the two experimental drugs. This difference in results can probably be explained by the different experimental techniques. In their work the heart was exposed and the atrial fibrillation was induced by the direct application of acetylcholine or aconitine. This extensive surgical manipulation may have caused a general weakening of the animal or an altered ionic concentration and have affected their results.

Ion Studies.—At no point in these studies is there any indication of a statistically significant ion flux. The ranges for the ion values obtained are presented in Table III.

These findings do not agree with those of the authors cited previously (6–12), however the methods used are probably not comparable.

Most work done previously was done on an isolated perfused preparation. Holland used an isolated perfused amphibian heart. The differences in experimental techniques and the differences between an isolated perfused amphibian heart and an intact mammalian heart are probably responsible for the differences between this work and his. Conn and Wood (8) used a dog's heart as an isolated perfused preparation. Again wide variations in experimental techniques are probably responsible for the difference in results.

There is a strong possibility that these ionic fluxes occur so rapidly that it is difficult to collect a blood sample at the exact peak of the flux. There is also a possibility that these ion changes are of such magnitude that the instrument used in this study would not be sensitive to them. Still another possibility for the discrepancy in these results and those of other workers is that the red blood cells may play a role in adjusting these fluxes. As the blood is coagulating, the red blood cells may tend to adjust the ionic imbalances back toward normal. Still another possibility for the inability to demonstrate these fluxes is that the kidney may make a rapid readjustment.

TABLE I.—FIBRILLATORY DURATION TIME

Dog No.	Quinidine, 5 mg./Kg. Before	Quinidine, 5 mg./Kg. After	Amodiaquin, 4 mg./Kg. Before	Amodiaquin, 4 mg./Kg. After	Primaquine, 4 mg./Kg. Before	Primaquine, 4 mg./Kg. After
1	5:20	2:20	4:45	4:15	5:20	3:10
2	3.47	2:27	2:25	1:35	4:07	1:56
3	3:28	1:34	2:30	1:10	2:25	1:10
4	6:00	3:05	5:25	3:10	2:30	1:07
5	4:12	2:19	3:51	2:00	4:03	3:00

TABLE II.—DIFFERENCE BETWEEN AVERAGE TIMES OF TABLE I

Dogs	Quinidine	Amodiaquin	Primaquine	Replication Totals
1	180	30	130	340
2	80	50	131	261
3	114	80	75	269
4	175	135	83	393
5	113	111	63	287
Total	662	406	482	1,550

Calculation of F Value

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Replication	4,113.5	4	1,033.35	0.556
Grand mean	160,166.6	1	160,166,66	86.19
Treatment	6,914.2	2	3,457.10	1.86
Error	14,865.8	8	1,858.23	
Total	186,080.0	15		

TABLE III.—RANGE OF ION VALUES IN MEQ./L.

	Low	High
Sodium		
Control	154.0	185.5
Neostigmine	137.5	187.0
Acetylcholine	130.0	215.0
Quinidine	125.0	182.2
Amodiaquin	142.0	170.5
Primaquine	145.0	186.5
Potassium		
Control	4.8	8.5
Neostigmine	4.9	9.9
Acetylcholine	4.2	10.2
Quinidine	5.4	7.6
Amodiaquin	6.13	9.0
Primaquine	4.4	6.5

SUMMARY AND CONCLUSIONS

An investigation of the antiarrhythmic properties of amodiaquin and primaquine has shown them to be as effective as quinidine in shortening the duration of experimentally induced atrial fibrillation but of stronger antiarrhythmic activity. The atrial fibrillation was induced by the injection of acetylcholine after the animal had been pretreated with the anticholinesterase agent, prostigmine.

This study has attempted to show the ionic changes caused by acetylcholine. Further, this study has attempted to show that these ionic fluxes are depressed by the antiarrhythmic drugs studied. No ionic fluxes are demonstrated at any point in this study so it could not be said that quinidine or related drugs had any demonstrable effect on the ionic concentration or ionic fluxes of the myocardial tissue.

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Stability Assays of Pharmaceutical Preparations by Quantitative Paper Chromatography

Steroid Hormones in Oil

By HENRY R. ROBERTS and KLAUS FLOREY

A quantitative paper chromatographic method has been developed which is routinely used as a stability assay for steroid esters having a 4-dehydro-3-keto configuration. The method involves: (*a*) a separation between the parent steroid ester and the free steroid, (*b*) the quantitative elution of the steroid ester from the chromatogram using an acidified methanolic solution of isonicotinic acid hydrazide, and (*c*) a spectrophotometric analysis of the eluate. Recoveries in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram. A detailed account of the procedure is presented using, as examples, the assay of 17-α-hydroxyprogesterone caproate and testosterone enanthate in oil formulations.

WHEN DEVELOPING analytical procedures for the determination of active ingredients in pharmaceutical preparations, it is necessary to make a distinction between content and stability assays. For a content assay, any characteristic property of the molecule amenable to convenient quantitation can be used. A stability assay, on the other hand, has to show whether or not the amount originally put into a formulation has undergone changes which, with time, will alter its pharmacological potency. A stability assay must, therefore, measure either the intact active ingredient or its breakdown products.

In cases where breakdown products are either difficult to measure chemically because they closely resemble the active ingredient or are altogether unknown, we have found it convenient to follow the stability of formulations by measuring the active ingredients by quantitative paper chromatography. In quite a number of cases, this method reduces the need for elaborate extraction procedures, especially when two or more similar components have to be separated. It also eliminates the cumbersome task of preparing, for comparison, blank formulations which contain all the ingredients except the active one.

Previous workers (1–3) have described the application of quantitative paper chromatography for the assay of various pharmaceuticals. We have adapted quantitative paper chromatography specifically to measure product stability of a great variety of pharmaceutical preparations. In this paper we wish to report on techniques which permit the quantitation of 4-dehydro-3-keto steroids with isonicotinic acid hydrazide (4). The examples chosen are the quantitative determination of two steroid hormones, testosterone enanthate and 17α-hydroxyprogesterone caproate in oil formulations.

Received October 20, 1961, from the Squibb Institute for Medical Research, New Brunswick, N. J.
Accepted for publication January 12, 1962.

EXPERIMENTAL

The paper chromatographic procedure involves, essentially, two steps: (*a*) the separation of the active component from its hydrolytic product and (*b*) the elution of the parent compound from the chromatogram followed by a quantitative spectrophotometric analysis of the eluate.

Preparation of Standards.—The reference standard solution should contain 1 mg. of the steroid hormone per ml. of solution. In a tared volumetric flask, the appropriate amount of steroid is dissolved in the smallest volume of N,N-dimethylformamide. Then methyl isobutyl ketone is added to the mark. The reference standard material should be from the same lot as the formulation undergoing analysis.

Preparation of Sample.—The amount of sample which will yield, based on theory, 1 mg. of the hormone per ml. of solution is weighed into a tared volumetric flask, dissolved, and diluted to the mark with methyl isobutyl ketone.

Filter Paper.—Whatman No. 1, $5^3/_4$ in. × 18 in., shape ERS, with 5 cut-outs. Prior to spotting, the papers are immersed for a minimum of 30 minutes in each of two 95% ethanol washes in order to minimize the concentration of ultraviolet light absorbing impurities in the paper. A large Pyrex baking dish can be used satisfactorily to wash 20 folded sheets at one time. The strips are folded but not creased. Following the second alcohol wash, the strips are air dried.

Solder is used to mark the strips with two lines prior to the spotting step. The origin line is $3^1/_4$ in. from the top.

The second line, $2^1/_4$ inches from the top, serves as a folding guide, providing the strip with a $2^1/_4$-inch tab which is inserted into the solvent trough.

Developing Solvent.—Methylcyclohexane saturated with diethylene glycol monoethyl ether[1] (DGME) (5).

[1] 2-(2-Ethoxyethoxy)ethanol, marketed as a Carbitol by Carbide and Carbon Chemical Co.

Chromatographic Chamber.—Any conventional chamber used for descending paper chromatography is suitable.

Eluting Solution.—A 1-Gm. quantity of isonicotinic acid hydrazide is dissolved in 1 L. of absolute methyl alcohol containing 1.25 ml. of concentrated hydrochloric acid.

Paper Chromatographic Procedure.—Two chromatograms are run per sample. The filter paper contains six 3/4-inch strips which are spotted as illustrated in Fig. 1.

Blow-out pipets, 0.1 ml. capacity, graduated in 0.01 ml., are used to apply the standards and the sample to the paper. The solutions are applied in aliquots of approximately 0.005 ml. so that the area of application remains small. A stream of warm air is directed on the spot after each aliquot is applied. The air is warmed by passing it through heated coiled copper tubing.

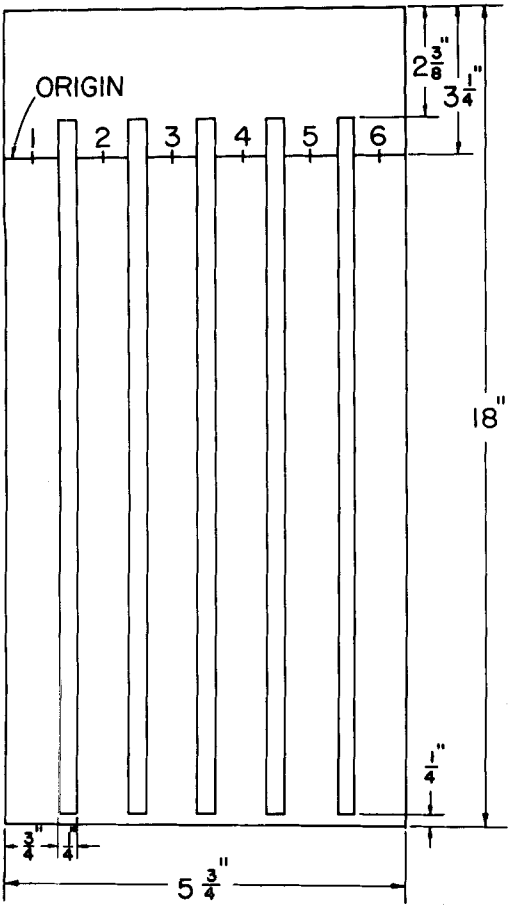


Fig. 1.—Diagram of spotting arrangement for assay of sex hormones in parenteral solutions.

	No. 1 Chromatogram	No. 2 Chromatogram
1	= Standard, 0.05 ml.	Standard, 0.05 ml.
2	= Standard, 0.10 ml.	Standard, 0.10 ml.
3	= Sample, 0.05 ml.	Sample, 0.10 ml.
4	= Sample, 0.05 ml.	Sample, 0.10 ml.
5	= Sample, 0.05 ml.	Sample, 0.10 ml.
6	= Paper blank	Paper blank

TABLE I.—PAPER CHROMATOGRAPHIC CONDITIONS FOR ASSAY OF SEX HORMONES[a]

Compound	Time, hr.
Testosterone enanthate	2 1/2
17α-Hydroxyprogesterone caproate	4

[a] Paper impregnating solution, 30% DGME in chloroform; developing solvent, methylcyclohexane saturated with DGME.

A spotting board, Fig. 2,[2] while not absolutely essential, offers a distinct advantage. It not only provides a support for the paper but it also holds the paper, by vacuum, in a fixed position. This permits spotting with one hand while the other hand is free to dry the spot after each application.

The spotted strips are impregnated with DGME by dipping them into the appropriate solution of DGME in chloroform, see Table I. The short end of the strip (3 1/4 inches from the origin) is dipped first, just up to the origin, and blotted lightly. The remainder of the strip is then passed through the impregnating solution with a rapid pass over the origin. In this manner, the entire strip, including the spotted origin, becomes impregnated with DGME. Since the extent of DGME impregnation will influence the mobility of the steroid being assayed, it is important that the DGME in chloroform solution be prepared accurately. Also, since evaporation of chloroform will alter the concentration of DGME, it is best if approximately 100 ml. of dipping solution is used to impregnate two to three chromatograms.

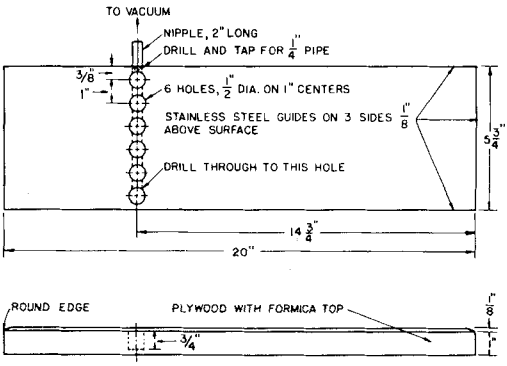


Fig. 2.—Board for spotting filter paper strips.

The impregnated strips are hung in a hood until the odor of chloroform can no longer be detected, and then they are placed in the chamber, the bottom of which is covered with DGME saturated with methylcyclohexane. The tab ends of the strips are placed into the troughs which contain the developing solvent, methylcyclohexane saturated with DGME. Following solvent development, Table I, the strips are removed from the chamber and dried in a mechanical convection oven for 20 minutes at 90°.

The positions of the steroids on the chromatograms are located by examining the strips through a fluorescent paddle over an ultraviolet light source.[3]

[2] The board shown here is a modification of the original design for which we are indebted to John M. West of these laboratories.

[3] The simple and efficient U.V. light source and paddle described here are a modification of the original design of Drake, *et al.* (6). We are indebted to R. W. Thoma and J. R. Gerke of these laboratories for these modifications.

The locations of the steroids are seen as black areas since they absorb the U.V. radiation and block the fluorescence. The source of ultraviolet radiation is a wooden box (8 in. × 8 in. × 21 in.) containing a 15-watt germicidal lamp (General Electric, 17 inch, No. G 1578) in the bottom and covered on top with three $6^1/_2$ in. sq. pieces of red-purple Corex A filters (No. 9863, Corning Glass Works, Corning, N. Y.).

The paddle is made from $^1/_8$ in. clear plexiglas and measures 7 in. square with a $1^1/_2 \times 4$ in. handle. It is coated with a thin layer of green fluorescing zinc silicate as follows: 6 ml. of collodion is measured into a 50-ml. glass-stoppered graduated cylinder and diluted to 22 ml. with acetone. To this is added 2 Gm. of zinc silicate phosphor (No. 609, E. I. DuPont de Nemours and Co., Towanda, Pa.). The mixture is shaken thoroughly and poured onto the paddle. The paddle is tipped in all directions so that the whole surface is evenly covered with a thin layer. Excess material is dripped off the paddle in the process of distributing it evenly.

The areas of the strips containing the steroids are marked with a solder pencil, cut out and folded, and placed in 50-ml. Erlenmeyer flasks. A paper blank, equal in area to the standard and sample segments, is included for each chromatogram (see Fig. 1). A piece of plywood mounted with 48 flask clamps (No. XCO050, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) holds the flasks during the elution step. A 10-ml. quantity of the isonicotinic acid hydrazide solution is added to each flask, the flasks covered with aluminum foil to prevent the evaporation of the methanol, and then shaken for 60 minutes on a reciprocating shaker (130 strokes per min.). A variable reciprocating laboratory shaker manufactured by Eberbach (No. G-21827, Emil Greiner Co.) is ideal for this step. The 60-minute shaking period is necessary for (*a*) the elution of the steroid and (*b*) the reaction, at room temperature (*ca.* 22°), between the eluted 4-dehydro-3-keto steroid and the isonicotinic acid hydrazide reagent to form a yellow hydrazone.

The solution is then decanted into a 1-cm. Corex cell and the absorbance is read at 415 mμ, using a Beckman DU spectrophotometer. Absolute methanol is used as the reference solution. The standard and sample readings are corrected for cell readings and for the paper blank.

Calculations.—The absorbance readings are recorded on a data sheet which contains all the information necessary to calculate the concentration of steroid in the sample. Table II gives the data obtained in a typical analysis.

For each of the two chromatograms, absorbance readings are obtained for the standard at two levels and for the sample at one level, in triplicate. The absorbance readings of the two standards for each of the chromatograms are converted to the equivalent absorbance for the 0.1 ml. standard concentration and averaged. The three absorbance readings of the sample are converted to 0.1 ml. readings and averaged.

The concentration of the steroid in the sample is calculated from the following equation

$$\text{mg. of steroid per ml. formulation} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where A = average absorbance of 10 ml. eluate of 0.1 ml. chromatographed sample, B = weight in mg. of 0.1 ml. chromatographed standard, C = volume in ml. of sample dilution, D = specific gravity of formulation, E = average absorbance of 10 ml. eluate of 0.1 ml. chromatographed standard, F = weight in Gm. of sample, and G = volume (0.1 ml.) of chromatographed sample.

The final concentration of the steroid in the sample is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—Table II, chromatogram No. 1

$$\text{mg. testosterone enanthate per ml.} = \frac{0.119 \times 0.1048 \times 100 \times 1.0127}{0.180 \times 0.1752 \times 0.1} = 400$$

RESULTS AND DISCUSSION

Recoveries in excess of 95% must be obtained if any analytical method is to serve as an effective stability assay. The accuracy and precision of the method were established by determining the 17α-hydroxyprogesterone caproate content of a Delalutin 2X preparation. Ten aliquots were weighed and diluted; two chromatograms were run per aliquot. The average value of 253 mg./ml. obtained from 10 individual assays (Table III) differed from the theoretical value by 1.2%. In every instance, no individual assay differed from the theoretical value by more than 3.2%, well below the 5% maximum limit placed on the assay. The standard deviation, 3.74 mg./ml., indicates that the assay can be repeated with good precision.

Accuracies of this magnitude are obtained since the standards and sample are run on the same chromatogram in replicate and both are exposed simultaneously to the same chromatographic and assay conditions. Table IV contains additional data indicating that assays with recoveries in excess of 95% are obtained consistantly.

The hormone preparations described here are dissolved in sesame or castor oil. They contain, in addition, some combination of benzyl alcohol, benzyl benzoate, and chlorobutanol.

Not only does the paper chromatographic procedure separate the active ingredient from its hydrolytic product but it achieves this without the need of any elaborate extraction procedure. A simple dilution prior to spotting is all the sample preparation required.

A marked separation is achieved on the chromatogram between the parent steroid esters and the free steroids. Testosterone and 17α-hydroxyprogesterone remain near the origin while the esters migrate down the strip. While the intact ester is measured, the procedure also permits a visual identification and, if necessary, quantitative measurement of the hydrolytic product. However, the stabilities of the steroid esters in the oil formulations described here are excellent and no breakdowns have been observed upon prolonged storage at elevated temperatures, Table V.

The other components in the formulation do not interfere. The only possible interference originated with the benzyl benzoate. This compound is a U.V. absorber which has a chromatographic mobility similar to the steroids being assayed. While a clear

TABLE II.—DATA SHEET FOR ASSAY OF TESTOSTERONE ENANTHATE IN DELATESTRYL 2X

Project: Te.204-53 Delatestryl 2X | Filter Paper: EtOH Washed Finger Strips | Wavelength: 415 mμ
Sample: Stability -50°C, 1 month | Developing Solv.: Methylcyclohexane saturated with Carbitol | Slit Width: 0.06
Assay: Testosterone Enanthate | Developing Time: 2-1/2 hrs. | Cell Corrections:
Sample Wt.: 175.2 mg./100 ml. | Drying Cond.: 20 min. 90°C. | No.1: +0.001
Sp. Gr. Sa.: 1.0127 | Eluting Solution: 10 ml. Hydrazid | No.2: +0.001
Wt. Standard: 26.2 mg./25 ml. | Eluting Time: 60 Min. | No.3: +0.002
Corr. Solvent: Abs. Methanol

Chrom.	Flask No.	Cell No.	Spot	O.D.	Corrected O. D. Cell.	Corrected O. D. Blank	O.D. St.=0.10 ml. Sa.=0.10 ml.	O.D. (Av.) St.=104.8γ Sa.=0.10 ml.	Conc. mg./ml. Formulation
1	1	1	Std.=0.05 ml.	0.142	0.141	0.089	0.178	0.180	
	2	2	Std.=0.10 ml.	0.234	0.233	0.181	0.181		
	3	3	Sa.=0.05 ml.	0.113	0.111	0.059	0.118		
	4	1	Sa.=0.05 ml.	0.112	0.111	0.059	0.118	0.119	400
	5	2	Sa.=0.05 ml.	0.113	0.112	0.060	0.120		
	6	3	Paper Blank	0.054	0.052	-			
2	7	1	Std.=0.05 ml.	0.143	0.142	0.088	0.176	0.178	
	8	2	Std.=0.10 ml.	0.236	0.235	0.181	0.181		
	9	3	Sa.=0.10 ml.	0.169	0.167	0.113	0.113		
	10	1	Sa.=0.10 ml.	0.169	0.168	0.114	0.114	0.115	392
	11	2	Sa.=0.10 ml.	0.173	0.172	0.118	0.118		
	12	3	Paper Blank	0.056	0.054				

Average: 396 mg./ml.

separation between the steroid and benzyl benzoate is not obtained, its presence is negated by reacting the steroid with isonicotinic acid hydrazide.

The use of acidified alcoholic isonicotinic acid hydrazide solution as a colorimetric reagent and as a paper chromatographic spray reagent for the detection of 4-dehydro-3-keto steroids have been described by a number of investigators (4, 7, 8). The manner in which it is utilized as a simultaneous eluant and colorimetric reagent as reported here has not, to the authors' knowledge, been described previously.

The absorption spectra of the isonicotinyl hydrazones of testosterone enanthate and 17α-hydroxyprogesterone caproate are similar to the spectrum given by Umberger (4) for the hydrazone of testosterone with a maximum at 380 mμ. The absorbances of the eluates can be measured in two different ways, both giving equally reliable results (Table VI). Absorbances of the eluates can be read at 380 mμ using the hydrazide reagent as a reference solution. At this wavelength the paper blank readings are extremely low. However, due to evaporation of the solvent, the concentration of the reference solution changes very rapidly and has to be replaced after every three readings. This becomes tedious and time consuming when the absorbances of a large number of eluates have to be read. There-

TABLE III.—ACCURACY AND PRECISION OF THE PAPER CHROMATOGRAPHIC DETERMINATION OF 17-α-HYDROXYPROGESTERONE CAPROATE IN DELALUTIN 2X

Aliquot No.	Found, mg./ml.	Deviation from Theory,[a] %
1	254	+1.6
2	258	+3.2
3	249	−0.4
4	258	+3.2
5	248	−0.8
6	254	+1.6
7	249	−0.4
8	251	+0.4
9	256	+2.4
10	251	+0.4
Average	253	+1.2
Standard deviation	3.74	
Coefficient of variation, %	1.48	

[a] Theoretical value, 250 mg./ml.

TABLE IV.—PAPER CHROMATOGRAPHIC ANALYSES OF SEX HORMONES IN PARENTERAL DELAHORMONE PREPARATIONS

Active Ingredients per ml.	Found, mg./ml.	Deviation From Theory, %
Testosterone enanthate,[a] 200 mg.	200	0
	206	+3.0
	198	−1.0
	196	−2.0
Testosterone enanthate,[b] 400 mg.	407	+1.8
	410	+2.5
	405	+1.2
	403	+0.8
17α-Hydroxyprogesterone caproate,[c] 125 mg.	120	−4.0
	126	+0.8
17α-Hydroxyprogesterone caproate,[d] 250 mg.	255	+2.0
	257	+2.8
	256	+2.4
	255	+2.0
Testosterone enanthate 180 mg.; Estradiol valerate,[e, g] 8 mg.	178	−1.1
	181	+0.5
17α-Hydroxyprogesterone caproate 250 mg.; Estradiol valerate,[f, g] 5 mg.	254	+1.6
	250	0
	252	+0.8

Squibb trade names: [a] Delatestryl. [b] Delatestryl 2X. [c] Delalutin. [d] Delalutin 2X. [e] Deladumone 2X. [f] Deluteval 2X. [g] Quantitative paper chromatographic assay of estrogens described in paper II of this series.

TABLE V.—PAPER CHROMATOGRAPHIC ANALYSES OF SEX HORMONES IN OIL UNDERGOING STABILITY STUDIES

Sample No.	Active Ingredient per ml.	Time, Mo.	Temperature, °C. 5	22	40	50
			Found, mg./ml.			
1	Testosterone enanthate, 180 mg.	12	...	181	187	185
2	Testosterone enanthate, 200 mg.	12	211	214	214	209
3	Testosterone enanthate, 200 mg.	12	206	196	205	199
4	Testosterone enanthate, 200 mg.	24	...	195	209	195
5	17α-Hydroxyprogesterone caproate, 125 mg.	12	126	125	132	129
6	17α-Hydroxyprogesterone caproate, 125 mg.	12	126	127	128	132
7	17α-Hydroxyprogesterone caproate, 125 mg.	18	...	126	128	129
8	17α-Hydroxyprogesterone caproate, 250 mg.	6	258	253	257	254
9	17α-Hydroxyprogesterone caproate, 250 mg.	6	...	257	256	251
10	17α-Hydroxyprogesterone caproate, 250 mg.	12	255	257	256	255

TABLE VI.—COMPARISON OF TESTOSTERONE ENANTHATE CONTENT CALCULATED FROM ELUATE ABSORBANCES

Chromatogram	380 mμ[a] Found, mg./ml.	380 mμ[a] Deviation from Theory,[c] %	415 mμ[b] Found, mg./ml.	415 mμ[b] Deviation from Theory, %
1	394	−1.5	406	+1.5
2	403	+0.75	396	−1.0
3	416	+4.0	410	+2.5
4	389	−2.75	405	+1.25
5	416	+4.0	414	+3.5
Average	404	+1.0	406	+1.5

[a] Read against the isonicotinic acid hydrazide reagent; fresh reagent blank for every three eluate readings. [b] Read against absolute methanol; same blank for all eluate readings. [c] Theoretical value, 400 mg./ml.

fore, as described in the experimental part, the absorbances of the eluates are read at 415 mμ against methanol as the reference solution. At this wavelength, lower paper blank readings ($A \backsim 0.050$) are obtained than at 380 mμ ($A \backsim 0.200$). The high paper blank at 380 mμ is primarily due to the absorbance of excess hydrazide in the eluates (4).

The generally accepted procedure when working with Zaffaroni-type chromatographic systems is to impregnate the paper with the stationary phase prior to spotting the steroidal solution. For quantitation, spotting the paper prior to impregnation is more expeditious since the spotting-dipping sequence provides for better control over the uniformity of the stationary phase. Furthermore, papers can be spotted well in advance of chromatography. No distortion or diffusion of the zones has been noted when the impregnation of the spotted origin is done rapidly, as described.

The use of slotted sheets of filter paper offers a number of apparent advantages. The steroids corresponding to the standard and sample applications are confined in their solvent migration to their own 3/4-in. wide strips. Lateral diffusion is suppressed and contamination from adjacent spots can never occur. Also, the area of each of the strips containing the steroid, as well as the paper blank segment, are at all times equal since only the north-south boundaries have to be ascertained.

The design of the fluorescent screen as a paddle makes for easy marking of the steroid segments. The edges of the paddle are used as guides while, at the same time, assuring that all the absorption is confined to the outlined zones.

SUMMARY

1. A quantitative paper chromatographic assay has been developed for the assay of 4-dehydro-3-keto steroids using an acidified methanolic solution of isonicotinic acid hydrazide.

2. The procedure is used routinely to measure the stability of a number of sex hormones in oil preparations. The assay of 17α-hydroxyprogesterone caproate and testosterone enanthate are described in detail.

3. The method is simple and accurate and permits the simultaneous visual identification and, if necessary, quantitative measurement of any hydrolytic product.

4. No extraction of the sex hormone is required. A simple dilution prior to chromatography is the only sample preparation required.

5. Accuracies in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram.

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Preparation and Pharmacological Properties of Some Benzyl Derivatives of Diphenylacetic Acid

By U. E. JOHNSEN, C. R. JACOBSON†, R. A. LaFORGE‡, and CALVIN HANNA§

Benzyl derivatives of diphenylacetic acid were prepared from the starting material 2-chlorodiphenylacetyl chloride. The compounds synthesized were the N-benzyl derivatives of 2-chloro-, 2-hydroxy-, 2-acetoxy-, 2-dimethylaminoethoxy-, and 2-benzylaminodiphenylacetamide; N-benzyl-N-methyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; O-benzyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; and the compound 2-benzylaminodiphenylacetamide. The compounds have limited aqueous solubility so the full extent of the possible pharmacologic actions could not be studied. These agents orally in doses of 1 Gm./Kg. produced no toxic symptoms but the N-benzyl-N-methyl-2-chloro- and the N-benzyl-2-hydroxy-diphenylacetamides inhibited the passage of a charcoal meal, indicating antispasmodic activity similar in potency to that of atropine. Intravenously, the compounds were without cardiovascular or respiratory effects. Intraperitoneally, N-benzyl-2-hydroxy and N-benzyl-2-acetoxy diphenylacetamides showed antipentamethylene tetrazol activity, and one of these also abolished the convulsive actions of electrical shock.

VARIOUS diphenylacetic acid derivatives have long been of interest as antispasmodic agents. The relatively recent literature indicates that certain compounds containing the diphenylacetyl moiety have also been found to possess sedative or anticonvulsant properties. For example, Billman, *et al.* (1, 2), have studied a series of substituted derivatives of 2-aminodiphenylacetamide possessing both anticonvulsant and antispasmodic activity. Among references in the patent literature, N-acetoxy (diphenylacetyl)acetamide (3) is claimed to be an antiepileptic without untoward effects and having extremely low toxicity in animals.

Also apparent from the recent literature is the frequency with which the benzyl group is found, usually as an amide or ester, in compounds with notable properties as sedatives or anticonvulsants. Kushner, *et al.* (4), also observed that many compounds containing a benzylamide moiety possessed pronounced anticonvulsant activity and their study of a variety of such substances resulted in the development of N-benzyl-β-chloropropionamide. N-Benzylmandelamide has

Received September 13, 1961, from the Research Laboratory of Bilhuber-Knoll Corp., Orange, N. J., and the Dept. of Pharmacology, University of Vermont, College of Medicine, Burlington.
Accepted for publication November 24, 1961.
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By U. E. JOHNSEN, C. R. JACOBSON†, R. A. LaFORGE‡, and CALVIN HANNA§

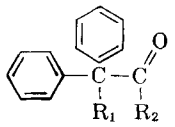
Benzyl derivatives of diphenylacetic acid were prepared from the starting material 2-chlorodiphenylacetyl chloride. The compounds synthesized were the N-benzyl derivatives of 2-chloro-, 2-hydroxy-, 2-acetoxy-, 2-dimethylaminoethoxy-, and 2-benzylaminodiphenylacetamide; N-benzyl-N-methyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; O-benzyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; and the compound 2-benzylaminodiphenylacetamide. The compounds have limited aqueous solubility so the full extent of the possible pharmacologic actions could not be studied. These agents orally in doses of 1 Gm./Kg. produced no toxic symptoms but the N-benzyl-N-methyl-2-chloro- and the N-benzyl-2-hydroxy-diphenylacetamides inhibited the passage of a charcoal meal, indicating antispasmodic activity similar in potency to that of atropine. Intravenously, the compounds were without cardiovascular or respiratory effects. Intraperitoneally, N-benzyl-2-hydroxy and N-benzyl-2-acetoxy diphenylacetamides showed antipentamethylene tetrazol activity, and one of these also abolished the convulsive actions of electrical shock.

VARIOUS diphenylacetic acid derivatives have long been of interest as antispasmodic agents. The relatively recent literature indicates that certain compounds containing the diphenylacetyl moiety have also been found to possess sedative or anticonvulsant properties. For example, Billman, *et al.* (1, 2), have studied a series of substituted derivatives of 2-aminodiphenylacetamide possessing both anticonvulsant and antispasmodic activity. Among references in the patent literature, N-acetoxy (diphenylacetyl)acetamide (3) is claimed to be an antiepileptic without untoward effects and having extremely low toxicity in animals.

Also apparent from the recent literature is the frequency with which the benzyl group is found, usually as an amide or ester, in compounds with notable properties as sedatives or anticonvulsants. Kushner, *et al.* (4), also observed that many compounds containing a benzylamide moiety possessed pronounced anticonvulsant activity and their study of a variety of such substances resulted in the development of N-benzyl-β-chloropropionamide. N-Benzylmandelamide has

Received September 13, 1961, from the Research Laboratory of Bilhuber-Knoll Corp., Orange, N. J., and the Dept. of Pharmacology, University of Vermont, College of Medicine, Burlington.
Accepted for publication November 24, 1961.
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TABLE I.—BENZYL DERIVATIVES OF DIPHENYLACETIC ACID



Compd. No.	R_1	R_2	M. P.,[a] °C.	Recrystn. Solvent	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
I	—Cl	—NH-Benzyl	109–110	Cyclohexane	75.01	5.44	4.21	75.05	5.49	4.10
II	—Cl	—N(CH_3)(Benzyl)	89–90	Cyclohexane	75.52	5.76	4.04	75.55	5.71	4.01
III	—Cl	—O—Benzyl	77–78	Ligroin	74.88	5.09	...	74.90	5.19	...
IV[b]	—OH	—NH—Benzyl	99–100	Cyclohexane	79.46	6.03	4.41	79.45	6.19	4.47
V	—OH	—N(CH_3)(Benzyl)	110.5–111.5	Aq. methanol	79.73	6.39	4.23	79.82	6.34	4.24
VI[c]	—OH	—O—Benzyl	74.5–75.0	Petr. ether	79.22	5.70	...	79.40	5.68	...
VII	—$OCOCH_3$	—NH—Benzyl	169.5–171.5	Isopropanol	76.86	5.89	3.89	76.85	5.97	3.90
VIII	—$OC_2H_4N(CH_3)_2$	—NH—Benzyl	57–58	Isopropanol	77.28	7.26	7.23	77.15	7.33	7.23
IX	—NH—Benzyl	—NH_2	163.0–164.5	Isopropanol	79.72	6.36	8.85	79.82	6.48	8.80
X	—NH—Benzyl	—NH—Benzyl	132.5–133.5	Isopropanol	82.72	6.45	6.89	82.65	6.45	7.05

[a] Melting points are not corrected. [b] Previously described by Lespagnol and Ponthieu (6) who report a melting point of 86°. [c] Previously described by Klinger and Standke (7), m.p. 75–76°.

been reported (5) to be an effective anticonvulsant against electroshock or pentylenetetrazol shock in rats.

These observations suggested the possibility that benzyl amides or esters of substituted diphenylacetic acids might well be active sedative or anticonvulsant agents and, further, might still retain some of the antispasmodic properties more commonly associated with derivatives of these acids.

The compounds of this series, together with pertinent chemical data, are listed in Table I. They were all prepared from 2-chlorodiphenylacetyl chloride which was synthesized from benzilic acid (1). Treatment of this acid chloride with ammonia, benzylamine, or N-benzylmethylamine gave 2-chlorodiphenylacetamide and the substituted 2-chlorodiphenylacetamides (I and II), while reaction with benzyl alcohol produced the benzyl ester (III). The 2-chloro derivatives (I, II, and III), were smoothly converted to the benzilamides (IV and V) or the benzilic acid ester (VI) by warming with aqueous acetone. N-Benzyl-2-chlorodiphenylacetamide (I) reacted with fused sodium acetate in glacial acetic acid to give N-benzyl-2-acetoxydiphenylacetamide (VII), while reaction with sodium 2-dimethylaminoethanolate gave N-benzyl-2-dimethylaminoethoxydiphenylacetamide (VIII). Treatment of 2-chlorodiphenylacetamide and the 2-chloroamide (I) with benzylamine gave the corresponding 2-benzylamino amides (IX and X).

EXPERIMENTAL

2-Chlorodiphenylacetyl Chloride.—This material was prepared by the improved procedure of Billman and Hidy (1) in yields of 70–80%, m.p. 49–50°.

N-Benzyl-2-chlorodiphenylacetamide (I).—Following the procedure of Billman, Ward, and Hidy (2), reaction of 2-chlorodiphenylacetyl chloride with benzylamine in anhydrous ether solution provided an 87% yield of pure N-benzyl-2-chlorodiphenylacetamide.

N-Benzyl-N-methyl-2-chlorodiphenylacetamide (II).—Substitution of N-benzyl-methylamine and dry benzene for benzylamine and anhydrous ether in the above procedure gave a 42% yield of the pure N-benzyl-N-methylamide.

Benzyl 2-chlorodiphenylacetate (III).—Addition of an ether solution of 2-chlorodiphenylacetyl chloride to a chilled ether solution of an equimolar quantity of benzyl alcohol, followed by stirring at room temperature for 24 hours, gave a 45% yield of the benzyl ester after concentration of the reaction solution and crystallization of the oily residue from ligroin.

Benzilic Acid Amides and Ester.—The 2-chlorodiphenylacetamides, (I and II), and the benzyl ester (III), were transformed into the corresponding benzilic acid amides and ester (IV, V, and VI) in 84, 75, and 68% yields, respectively, by refluxing the chloro compounds for 8–12 hours in a 1:3 acetone-water mixture. The products were isolated by concentration of the reaction mixtures and recrystallization of the solid residues from appropriate solvents.

N-Benzyl-2-acetoxydiphenylacetamide (VII).—This compound was prepared by reacting N-benzyl-2-chlorodiphenylacetamide with fused sodium acetate in glacial acetic acid according to the procedure of Emerson, *et al.* (8). The product was isolated in 48% yield by pouring the reaction mixture over ice and recrystallizing the precipitated solid several times from isopropanol.

N-Benzyl-2-dimethylaminoethoxydiphenylacetamide (VIII).—Dropwise addition of a solution of N-benzyl-2-chlorodiphenylacetamide in dry benzene to a benzene suspension of an equimolar quantity of sodium dimethylaminoethanolate, prepared from sodium and dimethylaminoethanol, was followed by refluxing of the reaction mixture for 12 hours. After filtering off the precipitated sodium chloride, the benzene solution was extracted with dilute

hydrochloric acid and the crude product was precipitated by addition of sodium hydroxide solution to the combined acidic extracts. Several recrystallizations from isopropanol afforded a 39% yield of the pure basic ether. The hydrochloride salt of the product decomposes at 192° after recrystallization from a mixture of methanol, isopropanol, and ether. It is not appreciably soluble in water.

2-Chlorodiphenylacetamide.—Addition of gaseous ammonia to a chilled ether solution of 2-chlorodiphenylacetyl chloride, following the method of Bickel (9), gave a pure product, melting at 118–119°, in 90% yield after recrystallization from toluene.

2-Benzylaminodiphenylacetamide (IX).—Benzylamine was reacted with 2-chlorodiphenylacetamide in benzene solution according to the procedure of Billman, Ward, and Hidy (2) to afford a 38% yield of pure product after several recrystallizations from isopropanol.

N-Benzyl-2-benzylaminodiphenylacetamide (X).—This compound was prepared by the above procedure from N-benzyl-2-chlorodiphenylacetamide and benzylamine in 45% yield.

PHARMACOLOGY

Pharmacologic studies were carried out on these compounds using dogs, cats, hamsters, and rats. The full extent of the possible actions of these chemicals could not be determined due to their restricted solubility in aqueous solutions. None of the agents were soluble to the extent of 1% in water or in 25% aqueous dimethylacetamide, and only compounds I and IV appeared to be soluble to the extent of 1% in ethylene glycol at 35°. Wherever possible, the compounds were suspended in water with gum acacia. Toxicity in rats could not be determined.

Doses of 1 Gm./Kg. given orally to rats produced no toxic symptoms. The influence of these agents on the intestinal motility of the small intestine was measured as the inhibition of passage of a charcoal meal, using the method of Macht and Gose. Each compound was suspended in an aqueous solution of 10% charcoal and 10% gum acacia. One milliliter of this suspension was given by stomach tube, and 1 hour later the rats were sacrificed, the intestines removed, and the distance traversed by the charcoal and the length of the intestines were measured. The ten controls averaged 69% with a standard deviation of ±6%. Atropine sulfate in doses of 0.1, 1.0, 2.0, and 3.0 mg./Kg. in 5 animals each was used as a standard of reference, giving the means and standard deviations as follows: 67±5, 62±5, 52±4, 50±4%, respectively. A dose of atropine *vs.* the percentage of the total distance the charcoal meal traversed curve was plotted. The effectiveness of the compounds tested was given in terms of equivalent effective doses of atropine. The test compounds were given in doses up to 0.75 Gm./Kg., and compounds II and VI influenced the intestinal motility. At this dose level, the activity of II and VI were equivalent to that of 1.0 and 0.2 mg./Kg. of atropine, respectively. At a dose level of 1.0 Gm./Kg. with these two agents, the activity of II was equivalent to 3.0 mg./Kg. of atropine and that of VI was equal to 2.0 mg./Kg. of atropine.

Blood pressure studies were made on the pentobarbitalized dog and cat following intravenous injections of 2.0 mg./Kg. of the chemicals in 25% ethanol in isotonic saline. There was only a minimal effect of these compounds on the mean arterial blood pressure; an equivalent amount of 25% ethanol in isotonic saline was used as a control and was found to be without effect on the blood pressure.

These compounds do not appear to be well absorbed orally because of lack of solubility and toxicity, so the intraperitoneal route of injection was used for the anticonvulsant testing. The agents were dissolved in hot 50% ethanol and cooled to 40° just before injection, and the animals in groups of 10 were tested 1 hour later. Control animals given 50% ethanol in saline in the volumes used in the experiments did not show central nervous system effects nor did they differ in convulsant thresholds when compared to saline-treated animals. Anticonvulsant studies were carried out first by the rat subcutaneous pentylenetetrazol test. This demonstrated the activity of the compound being tested to block the convulsions produced by 100 mg./Kg. pentylenetetrazol given subcutaneously. Phenobarbital sodium was used as the standard of comparison.

Due to limited solubility, compounds IV and VII were injected in doses of 100 mg./Kg., while the rest of the compounds were administered in doses of 40 mg./Kg. Only compounds IV and VII showed antipentylenetetrazol activity in doses of 40 mg./Kg. In doses of 100 mg./Kg., compound VII exhibited a minimal activity, while compound IV elicited an effectiveness similar to hypnotic doses of phenobarbital. The second method used was the supramaximal electrical shock test. This showed the ability of the compounds to abolish the hind limb extensor reflex produced by a current of 150 ma. delivered for 0.3 sec. to the brain via the ear electrodes, using the rat. Only compound IV, in doses of 100 mg./Kg., was effective in this respect by abolishing the hind limb extensor reflex in 4 out of the 10 animals.

The "wild" hamster is useful in determining sedative and tranquilizer effects, since the actions of the barbiturates and reserpine are easily differentiated when this animal is hungry and made angry by being picked up by the tail. Intraperitoneal injections of 40 mg./Kg. of these compounds had no effect on this animal.

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Analysis of Data on the Medicament Release from Ointments

By W. I. HIGUCHI

An analysis of some recently published data on the sodium radioiodide release from hydrophilic ointment bases is presented and discussed. The release dependence on time and the value for the effective diffusion coefficient are compared with theoretical predictions. The generally good agreement between theory and data demonstrates the potential usefulness of the theoretical relationships in both experimental design and data evaluation. The simplicity of some of the theoretical equations is indicated.

RECENT THEORETICAL investigations (1, 2) of drug release from ointments resulted in equations which were expected to be useful in describing ointment release rates. Because of the lack of availability of suitable data at the time, the applicability of these mathematical relationships to real situations was not experimentally demonstrated. Recently, however, Patel, Banker, and DeKay reported (3) results of some studies on sodium radioiodide release from hydrophilic ointment bases. Because an examination of their data with the appropriate theoretical relationships appeared to add to the better basic understanding of ointment behavior, this report was prepared for presentation at this time.

Higuchi deduced (1) the following equation for the amount of drug released from (one side of) a layer of ointment in which the drug is initially uniformly dissolved

$$Q = hC_0 \left[1 - \frac{8}{\pi^2} \sum_{m=0}^{\infty} \frac{1}{(2m+1)^2} \exp \left(- \frac{D(2m+1)^2 \pi^2 t}{4h^2} \right) \right] \quad \text{(Eq. 1)}$$

where Q = amount of drug released per unit area of application, h = thickness of layer, C_0 = initial concentration of drug in ointment, D = diffusion coefficient of drug in the ointment, t = time after application, m = integer, as indicated, goes from 0 to ∞.

The per cent released, R, is then simply

$$R = \frac{100Q}{hC_0} = 100 \left[1 - \frac{8}{\pi^2} \sum_{m=0}^{\infty} \frac{1}{(2m+1)^2} \exp \left(- \frac{D(2m+1)^2 \pi^2 t}{4h^2} \right) \right] \quad \text{(Eq. 2)}$$

Equation 1 is a solution to Fick's law of diffusion which is extensively described in a number of textbooks (4, 5). The main assumptions involved in Eq. 1, in practical language, are (*a*) that only a single drug species is important in the ointment, (*b*) that D must be constant with respect to both time and position in the ointment layer, (*c*) that only the drug is able to diffuse out of the layer, i. e., components of the vehicle cannot diffuse out (or evaporate), and finally (*d*) that the drug reaching the receptor side of the ointment layer is removed rapidly (means the same as the boundary condition: concentration of drug = 0 at receptor-ointment boundary for $t > 0$).

The experimental method described by Patel, Banker, and DeKay appears to conform approximately to all of the above requirements for the applicability of Eq. 1. Thus, a direct comparison of their data with Eq. 1 would seem to be justified. The difficulty with the idea of attempting a direct quantitative test is that the value of D is difficult to estimate for such complex systems as those studied by Patel, Banker, and DeKay. Therefore, the most profitable approach would be to consider first only the time-dependent part of the equation by selecting a value for D which gives the best overall fit for each set of data. Thus for each set of data one must choose the best single D value, which when placed into Eq. 2, will give the best agreement between the particular set of data and Eq. 2. This means effectively forcing a fit between data and theory at one point for each set of data. The agreement of the rest of the data for a particular set would constitute a test of the time-dependent portion of the theory. Then these chosen D values could be examined independently in light of the expected structure of the vehicle and with the mathematical relationships for D in heterogeneous systems (2).

TIME DEPENDENCE

In Fig. 1 representative sets of data taken from the work of Patel, Banker, and DeKay are given (circles). The different sets of data refer to sodium radioiodide release from bases[1] containing different surfactants or different amounts as indicated in the figure. The curves represent values predicted by Eq. 2 with a single value of D for each set of data.

The agreement of the time-dependent portion of Eq. 2 with the data is highly satisfactory indicating that the idealized conditions had been well approximated by the experimental method. These results support the assumptions (under assumption *d* above) that the Visking membrane in these experiments contributed negligibly to the release resistance and that stirring in the solution on the other side of the membrane was rapid enough to prevent buildup of a liquid diffusion layer of any

Received August 14, 1961, from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication September 25, 1961.
This material represents part of a paper presented before The Teachers Seminar on Pharmacy, University of Wisconsin, Madison, July 9–15, 1961.
The author is grateful to Professor DeKay for helpful correspondence.

[1] The reader is referred to the original work (3) for complete description of ointment bases. Except for the surfactant used, each base contained essentially the same ingredients and the same amounts of them as those in the official hydrophilic ointment U.S. P. XVI.

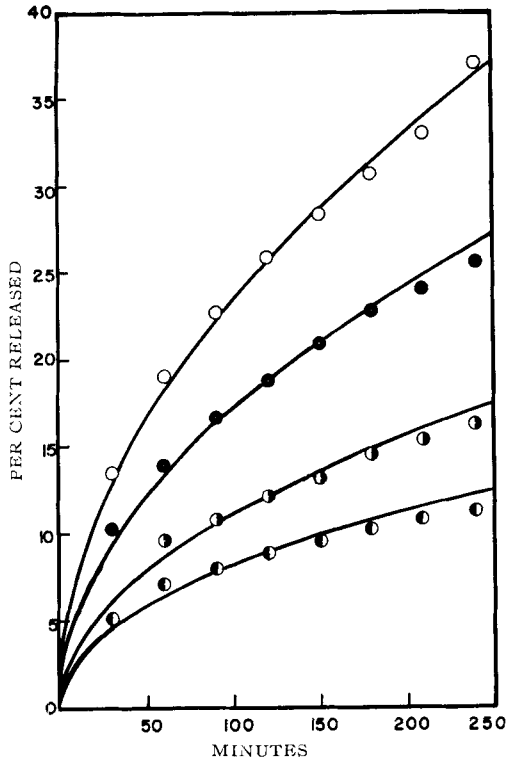


Fig. 1.—Comparison of data with theory for release from ointment base. Circles give data of Patel, Banker, and DeKay. Smooth curves obtained from Eq. 2 and a single adjustable parameter. ○, Sipon ES, 1%; ●, Sipon ES, 2%; ◑, Maprofix TLS, 3%; ◐, Catanac SN, 3%.

importance. If such were not the case, there would have been deviations of the data in the direction of less curvature than theory. Actually, the small deviations present are consistently in the opposite direction. The good agreement also supports the expected constancy of D (assumption b). This is reasonable in view of the diluteness of the sodium radioiodide in the vehicle.

It is worth while to point out at this time that for most practical applications of Eqs. 1 and 2, simplified forms of these equations may be used. When the per cent released, R, is not too large, i. e., for $R \lesssim 30\%$, it can be easily shown that

$$Q = 2\,C_0 \left(\frac{Dt}{\pi}\right)^{1/2} \qquad \text{(Eq. 3)}$$

and

$$R = 200 \left(\frac{Dt}{\pi h^2}\right)^{1/2} \qquad \text{(Eq. 4)}$$

These "square root" approximations should hold rather well for most studies of practical importance. The simplicity of Eqs. 3 and 4 makes the use of the theory very convenient.

EFFECTIVE OINTMENT DIFFUSION COEFFICIENTS

The values for the effective experimental diffusion coefficients derived from the data-theory fitting process described above are given in Table I.

Let us now see how these D values compare with the expected value based on our scanty knowledge

TABLE I.—EFFECTIVE DIFFUSION COEFFICIENTS OF SODIUM RADIOIODIDE IN OINTMENT BASES DETERMINED[a] FROM RELEASE DATA AT 37°

Base Designation	D (cm.^{2} sec.$^{-1}$)
Sipon ES, 1%	3.6×10^{-6}
Sipon ES, 2%	2.0×10^{-6}
Maprofix TLS, 3%	0.80×10^{-6}
Catanac SN, 3%	0.43×10^{-6}

[a] The value $h = 0.7$ cm. (3) was employed in these calculations.

of the detailed structure of these ointment bases. Since these hydrophilic bases are oil-in-water emulsions, the external phase is aqueous and presumably should represent about 40 to 50% of the total volume [corresponding to the 37% water and 12% propylene glycol by weight used (3)]. On the basis of what we know we could make both an upper limit estimate and a less exact "probable value" estimate of the effective diffusion coefficient.

For the upper limit estimate we assume that (a) sodium radioiodide is distributed only in the aqueous phase, that (b) it is able to diffuse only in the aqueous phase (i. e., zero diffusion coefficient in the internal phase), and that (c) the diffusion coefficient in the aqueous phase is the same as that in pure water. Note that assumption c makes this estimate an upper limit one. We may then write a relationship (2) for the effective diffusion coefficient in heterogeneous system where the internal phase may be considered to be impenetrable spheres

$$D_e = \frac{1.61 D_1}{3 - 1.39 V_1} \qquad \text{(Eq. 5)}$$

where D_e is the effective diffusion coefficient, D_1 is the diffusion coefficient in the external phase, and V_1 is the volume fraction of the external phase. Equation 5 appears to be a relatively good relationship for diffusion data (2) on suspensions of glass spheres, powders, sand, etc. For our calculation, if we take $V_1 \approx 0.45$, Eq. 5 gives $D_e \approx 0.7 D_1$. Now the literature value (6) for the diffusion coefficient of sodium iodide in water at 25° is 1.5×10^{-5} cm.^{2} sec.$^{-1}$. At 37° the value would be[2] about 1.9×10^{-5} cm.^{2} sec.$^{-1}$. Therefore, at 37°, $D_e \approx 1.3 \times 10^{-5}$ cm.^{2} sec.$^{-1}$, which is only about 3.6 times greater than the largest D value in Table I.

Now a "probable value" estimate can be made if it is permissible to assume that essentially all of the propylene glycol is in the aqueous phase. This consideration will make D_e smaller by about a factor of two[3] or so (with an uncertainty, say, about one-half of this).

The above analysis, although crude, clearly supports the expected mechanism of release. Sipon ES, 1%, represents a base with just about the maximum rate of release, i. e., further changes in surfactant or surfactant concentration should not increase the per cent release rate by a great deal. More independent information on these systems is needed before the dependence of D on surfactant type and surfactant concentration (see Table I) can be logically explained.

[2] Both Stokes-Einstein equation and data on similar systems predict this.

[3] Stokes-Einstein equation prediction (inverse proportionality with viscosity). Viscosity estimates inferred from other systems.

CONCLUSIONS

The analyses of the data of Patel, Banker, and DeKay by means of theory demonstrates the possible usefulness of the quantitative theory in future studies. Simple relations could be employed to help evaluate the proposed mechanisms. It would be of interest to investigate experimentally other types of ointments, e. g., the water-in-oil type, suspension type, bound drug type, etc., and compare results with appropriate theoretical relationships (1, 2).

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Synthesis of Some Monoamino Alkynes

By JOHN L. NEUMEYER† and JOSEPH G. CANNON‡

The preparation of a series of monoamino alkynes has been undertaken. A method of reduction of the substituted and unsubstituted quinolines to their corresponding tetrahydro derivatives is presented. Some of the monoamino alkynes showed weak fungicidal activity against *T. mentagrophytes*.

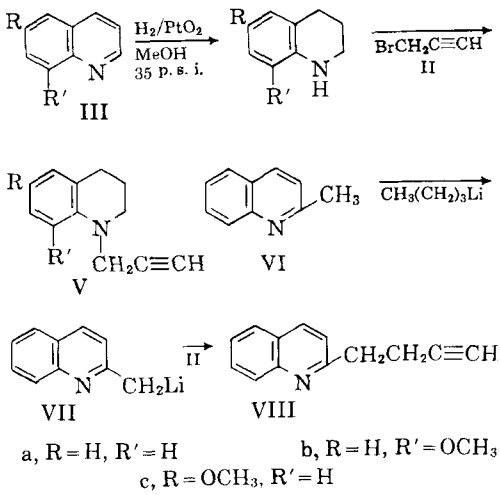
DURING THE COURSE of the investigation of a series of unsymmetrically alkylated acetylenic bis-quaternary ammonium compounds, it was necessary to prepare a series of monoamino alkynes (I)

$$RCH_2C{\equiv}CH$$
I

R = Pyrrolidino, diethylamino, 1,2,3,4-tetrahydroquinolino, 6-methoxy-1,2,3,4-tetrahydroquinolino, 8-methoxy - 1,2,3,4 - tetrahydroquinolino, 2 - quinolyl methyl

The structural similarity of certain of these intermediates to a series of 8-hydroxy-2-quinoline acrylic acids, prepared by Vaidya and Cannon (1), exhibiting fungicidal activity, prompted us to screen these compounds as potential fungicides. Preliminary screening against *T. mentagrophytes*, and *C. albicans* revealed that some of these compounds showed fungicidal activity against *T. mentagrophytes* at concentrations of 100–500 mcg./ml.

Propargyl bromide (II) was employed as the alkylating agent for the preparation of these compounds by the following routes.



a, R = H, R′ = H b, R = H, R′ = OCH_3

c, R = OCH_3, R′ = H

The catalytic reduction of quinoline and of the 6- and 8-methoxy-quinolines (III a, b, and c) was based on a method of Skita and Meyer (2) who reduced quinoline to decahydro- and tetrahydroquinoline with platinum chloride in glacial acetic acid. Employing platinum oxide (Adams) in methanol at 35 p.s.i., we successfully reduced the quinolines III a, b, and c to their respective tetrahydro derivatives in 80–96% yields. Propargyl bromide (II) was then treated with a 1-molar excess of the appropriate tetrahydroquinoline in isopropyl alcohol. The formation of quaternary propargyl halide salts was prevented by adding the propargyl bromide slowly to the amine. The alkynyl amines could be readily purified by fractional distillation

Received September 6, 1961, from the School of Pharmacy, University of Wisconsin, Madison 6.

Accepted for publication November 14, 1961.

Financial support from the National Institutes of Health (Fellowship HF9624) is gratefully acknowledged.

We are indebted to Dr. C. J. Sih for carrying out the fungicidal screening of the compounds reported herein.

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CONCLUSIONS

The analyses of the data of Patel, Banker, and DeKay by means of theory demonstrates the possible usefulness of the quantitative theory in future studies. Simple relations could be employed to help evaluate the proposed mechanisms. It would be of interest to investigate experimentally other types of ointments, e. g., the water-in-oil type, suspension type, bound drug type, etc., and compare results with appropriate theoretical relationships (1, 2).

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Synthesis of Some Monoamino Alkynes

By JOHN L. NEUMEYER† and JOSEPH G. CANNON‡

The preparation of a series of monoamino alkynes has been undertaken. A method of reduction of the substituted and unsubstituted quinolines to their corresponding tetrahydro derivatives is presented. Some of the monoamino alkynes showed weak fungicidal activity against *T. mentagrophytes*.

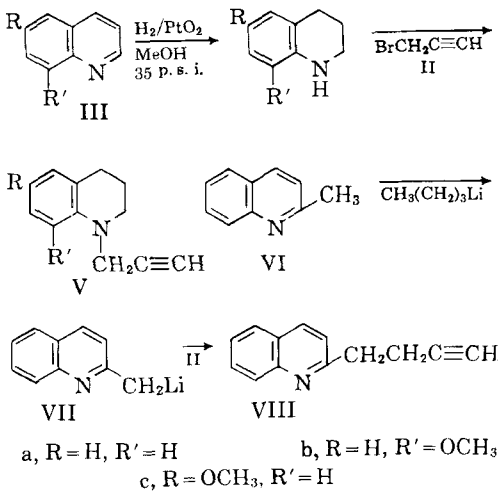
DURING THE COURSE of the investigation of a series of unsymmetrically alkylated acetylenic bis-quaternary ammonium compounds, it was necessary to prepare a series of monoamino alkynes (I)

$$RCH_2C{\equiv}CH$$
I

R = Pyrrolidino, diethylamino, 1,2,3,4-tetrahydroquinolino, 6-methoxy-1,2,3,4-tetrahydroquinolino, 8-methoxy - 1,2,3,4 - tetrahydroquinolino, 2 - quinolyl methyl

The structural similarity of certain of these intermediates to a series of 8-hydroxy-2-quinoline acrylic acids, prepared by Vaidya and Cannon (1), exhibiting fungicidal activity, prompted us to screen these compounds as potential fungicides. Preliminary screening against *T. mentagrophytes*, and *C. albicans* revealed that some of these compounds showed fungicidal activity against *T. mentagrophytes* at concentrations of 100–500 mcg./ml.

Propargyl bromide (II) was employed as the alkylating agent for the preparation of these compounds by the following routes.



a, R = H, R′ = H b, R = H, R′ = OCH_3

c, R = OCH_3, R′ = H

The catalytic reduction of quinoline and of the 6- and 8-methoxy-quinolines (III a, b, and c) was based on a method of Skita and Meyer (2) who reduced quinoline to decahydro- and tetrahydroquinoline with platinum chloride in glacial acetic acid. Employing platinum oxide (Adams) in methanol at 35 p.s.i., we successfully reduced the quinolines III a, b, and c to their respective tetrahydro derivatives in 80–96% yields. Propargyl bromide (II) was then treated with a 1-molar excess of the appropriate tetrahydroquinoline in isopropyl alcohol. The formation of quaternary propargyl halide salts was prevented by adding the propargyl bromide slowly to the amine. The alkynyl amines could be readily purified by fractional distillation

Received September 6, 1961, from the School of Pharmacy, University of Wisconsin, Madison 6.

Accepted for publication November 14, 1961.

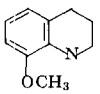
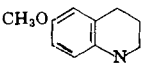
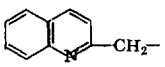
Financial support from the National Institutes of Health (Fellowship HF9624) is gratefully acknowledged.

We are indebted to Dr. C. J. Sih for carrying out the fungicidal screening of the compounds reported herein.

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TABLE I.—MONOAMINE ALKYNES
R—$CH_2C{\equiv}CH$

Compound	M.p. or B.p., °C.	n_D^{25}	Method	Yield, %	Formula	Analysis, % Calcd.	Analysis, % Found
$(CH_3CH_2)_2N$—	52/70 mm.	1.4291	B	75	...	...	...
Hydrochloride	194–195[a]	...	..	..	$C_7H_{14}ClN$	N, 9.50 Cl, 24.10	N, 9.18 Cl, 24.04
	92–94/150 mm.[b]	1.4622	B	78	...	...	...
Hydrochloride	151–151.5[c,d]	...	..	..	$C_7H_{12}ClN$	N, 9.63 Cl, 24.41	N, 9.43 Cl, 24.48
	92–94/0.3 mm.	1.5822	C	86	$C_{12}H_{13}N$	C, 84.20 H, 7.65 N, 8.17	C, 83.84 H, 7.60 N, 8.66
Hydrochloride	154–156[c,e]	...	..	..	$C_{12}H_{14}ClN$	N, 6.74 Cl, 17.08	N, 6.48 Cl, 16.90
	94–98/0.07 mm. 48–49[f]	... ...	C ..	75 ..	$C_{13}H_{15}NO$ ...	C, 77.60 H, 7.46 N, 6.97	C, 77.61 H, 7.34 N, 7.00
Hydrochloride	155–157[e]	...	..	..	$C_{13}H_{16}ClNO$	N, 5.89 Cl, 14.92	N, 5.56 Cl, 14.59
	100–102/0.01 mm. 45–46[g]	... ...	C ..	78 ..	$C_{13}H_{15}NO$ ...	C, 77.60 H, 7.46 N, 6.97	C, 77.41 H, 7.20 N, 7.58
Hydrochloride	156–158[e,f]	...	..	..	$C_{13}H_{16}ClNO$	N, 5.89 Cl, 14.92	N, 5.61 Cl, 14.57
	100–102/0.08 mm.	1.6042	D	33	$C_{13}H_{11}N$	C, 86.28 H, 6.08 N, 7.74	C, 86.61 H, 6.07 N, 8.52
Hydrochloride	144.5–146[g]	...	..	..	$C_{13}H_{12}ClN$	N, 6.47 Cl, 16.29	N, 6.77 Cl, 16.50

[a] Recrystallized from absolute ethanol-ether. [b] Biel (7) reports 74–77° (85 mm.). [c] Hygroscopic. [d] Recrystallized from *n*-butanol-Skelly B. [e] Recrystallized from isopropyl alcohol. [f] Recrystallized from Skelly A. [g] Recrystallized from ethanol-water. [h] Recrystallized from ethanol-Skelly B.

at reduced pressure. A 2-quinolyl derivative was prepared by treating *n*-butyl lithium with quinaldine (VI); the resulting 2-(lithio methyl) quinoline (VII) was condensed with an equimolar quantity of propargyl bromide, to yield 2-(3-butynyl) quinoline (VIII).

EXPERIMENTAL

All melting points were obtained in a Hershberg-type, silicone filled melting point apparatus equipped with Anschutz immersion thermometers. The samples were placed in the circulating silicone bath 10° below the melting points and heated at the rate of 1–2° per minute. Elemental analyses were performed by Drs. Weiler and Strauss, Oxford, England, and Huffman Microanalytical Laboratories, Wheatridge, Colo. Infrared spectra were determined on a Beckman IR-5 double-beam infrared recording spectrophotometer.

A. Substituted 1,2,3,4-tetrahydroquinolines

8 - Methoxy - 1,2,3,4 - tetrahydroquinoline.—8-Hydroxyquinoline was converted to 8-methoxyquinoline with dimethyl sulfate in 53% yield by the method of Vogel (3) m.p. 45–46° [Kaufman and Rothlin (4) reported 46–47°]. Eight grams (0.05 mole) of 8-methoxyquinoline in 100 ml. of methanol was hydrogenated in a Parr hydrogenation apparatus using 0.4 Gm. of platinum oxide as the catalyst; the calculated amount of hydrogen was absorbed in 50 minutes. The product was isolated by removing the catalyst by filtration, evaporating the solvent under reduced pressure, and fractionally distilling the residue, b.p. 94–98° (0.75 mm.), yield 7 Gm. (87%); n_D^{26} 1.5846.

Anal.—Calcd. for $C_{10}H_{13}NO$: C, 73.60; H, 7.98; N, 8.60. Found: C, 73.82; H, 7.83; N, 9.02.

The benzenesulfonamide of this secondary amine was prepared by shaking 0.5 Gm. of the amine in 25 ml. of 5% solution of sodium hydroxide and 1 Gm. of benzenesulfonyl chloride. An oil separated which, after standing for several days, crystallized. The yellow needles were recrystallized several times from 95% ethanol to yield a white crystalline product, m.p. 91.8–92.4°.

Anal.—Calcd. for $C_{16}H_{17}NO_3S$: C, 63.29; H, 5.65; N, 4.62. Found: C, 63.07; H, 5.30; N, 4.90.

6-Methoxy-1,2,3,4-tetrahydroquinoline (Thalline).—Twenty-five grams (0.157 mole) of 6-methoxyquinoline (Eastman white label) in 100 ml. of methanol was hydrogenated in a Parr hydrogenation apparatus using 0.4 Gm. of platinum oxide as the catalyst. The calculated amount of hydrogen was absorbed in 19 hours. The product was purified by vacuum distillation to give 24 Gm. (96% yield) of a pale yellow oil b.p. 84–85° (0.01 mm.). The oil solidified on cooling and the compound was recrystallized from 95% ethanol, m.p. 40–42°. [Cromwell (5) using a copper chromite catalyst under a pressure of 1800 lbs./sq. in. at 180° reported a product with a m.p. 42–43° in 93% yield.]

B. 3-Amino-1-propynes

To 1.0 mole of secondary amine in 150 ml. of anhydrous ethyl ether was added, with stirring,

59.5 Gm. (0.5 mole) of propargyl bromide (Aldrich Chemical Co.). The mixture was stirred at reflux for 12 hours. The ether solution was decanted from the oily amine hydrobromide layer, and the latter was extracted three times with ether. The ether was evaporated from the combined ether extracts and the residue was fractionated at reduced pressure (see Table I). The hydrochloride salts were prepared by passing dry hydrogen chloride through ether solutions of the amines. The precipitated salts were collected on a filter, washed with ether and were recrystallized (see Table I).

C. Aminoalkynyltetrahydroquinolines

To 0.18 mole of a freshly distilled 1,2,3,4-tetrahydroquinoline in 35 ml. of isopropyl alcohol was added 10.7 Gm. (0.09 mole) of propargyl bromide over a period of 30 minutes with stirring. The reaction mixture was refluxed 2 hours, then the mixture was cooled to 4° and was allowed to stand for 12 hours. The precipitate of the 1,2,3,4-tetrahydroquinoline hydrobromide was removed by filtration, and the filtrate was subjected to fractional distillation (see Table I).

The hydrochloride salts were prepared by passing dry hydrogen chloride through an ether solution of the amine. The precipitated salts were collected on a filter, washed with ether, and were recrystallized (see Table I).

D. 2-(3-Butynyl)quinoline

2-(Lithio methyl)-quinoline was synthesized by the method of Cannon (6) for the preparation of 1-(lithio methyl) isoquinoline. To the ethereal solution containing 0.1 mole of 2-(lithio methyl)-quinoline was added, in a slow stream and with stirring, a solution of 11.9 Gm. (0.1 mole) of propargyl bromide in 100 ml. of anhydrous ether. The solution turned a deep purple color and a moderate boiling ensued. As additional organic halide was added the purple color became fainter and finally disappeared. Stirring was continued overnight at room temperature, during which time a brown precipitate formed. Stirring was continued while 100 ml. of distilled water was slowly added. The aqueous layer was removed and discarded and the ethereal solution which also contained a considerable quantity of a brown precipitate was filtered and washed several times with water. After drying with anhydrous magnesium sulfate and filtering, the ether was removed from the filtrate on a steam bath and the brown-black oily residue was distilled under reduced pressure (see Table I). $\lambda_{max.\mu}^{film}$ 3.05 (=CH stretching), m 4.70 (—C≡C— stretching.)

E. Fungicidal Activity

Fungicidal activity was evaluated by a serial dilution procedure, utilizing Sabouraud's medium. The tubes were incubated at 25°; turbidimetric and growth readings were taken after 48 hours.

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Some 1,2,3,4-Benzothiatriazine 1,1-Dioxides

By SCOTT J. CHILDRESS

1,2,3,4-Benzothiatriazine 1,1-dioxide analogs of chlorothiazide have been prepared and tested for diuretic activity.

NOVELLO and Sprague (1) in 1957 reported the synthesis and diuretic activity of 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine 1,1-dioxide (chlorothiazide). Since then numerous analogs have been described (2). There are no specific structural requirements for activity except for the presence of a sulfamyl group and even this can be substituted (3). It seemed to be of interest to prepare some substituted 1,2,3,4-benzothiatriazine 1,1-dioxides and examine them for diuretic activity.

Received October 18, 1961, from the Research and Development Division, Wyeth Laboratories, Inc., Radnor, Pa.
Accepted for publication October 25, 1961.

In order to isolate stable compounds, it was necessary to have an alkyl group in the 2-position. For convenience in synthesis, the 7-sulfamyl group was also substituted by an alkyl group. It did not seem that such substitution would destroy any potential activity in the benzothiatriazine 1,1-dioxides, for 5-chloro-2,4-bis-(methylsulfamyl)aniline, an intermediate, is reported to have diuretic properties (4). Also, methyl and isopropyl substituents in sulfamyl groups are removed metabolically in some cases (5).

The desired benzothiatriazine 1,1-dioxides were prepared by treatment of the appropriate 5-chloro-2,4-bis-(alkylsulfamyl)aniline with nitrous acid (6). The intermediates are listed in Table I and the products in Table II. The benzothiatriazine 1,1-dioxides proved to have statistically significant diuretic activity in rats (7).

59.5 Gm. (0.5 mole) of propargyl bromide (Aldrich Chemical Co.). The mixture was stirred at reflux for 12 hours. The ether solution was decanted from the oily amine hydrobromide layer, and the latter was extracted three times with ether. The ether was evaporated from the combined ether extracts and the residue was fractionated at reduced pressure (see Table I). The hydrochloride salts were prepared by passing dry hydrogen chloride through ether solutions of the amines. The precipitated salts were collected on a filter, washed with ether and were recrystallized (see Table I).

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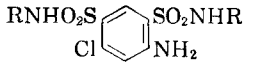
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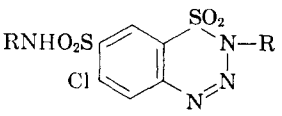
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TABLE I.—INTERMEDIATES FOR BENZOTHIATRIAZINE 1,1-DIOXIDES



R	M.P., °C.	Formula	Analysis, % Calcd. C	H	N	Found C	H	N
CH_3—	181–182	$C_8H_{12}ClN_3O_4S_2$	30.62	3.85	13.39	30.55	3.83	13.28
i-C_3H_7—	232–234	$C_{12}H_{20}ClN_3O_4S_2$	38.96	5.45	11.36	39.34	5.46	11.24
$C_6H_5CH_2$—	146–147	$C_{20}H_{20}ClN_3O_4S_2$	51.55	4.33	9.02	51.85	4.41	8.91
Cyclo-C_6H_{11}—	192–194	$C_{18}H_{28}ClN_3O_4S_2$	48.04	6.27	9.34	48.20	6.25	9.18

TABLE II.—BENZOTHIATRIAZINE 1,1-DIOXIDES



R	M.P., °C.	Formula	Analysis, % Calcd. C	H	Cl	S	Found C	H	Cl	S
CH_3—	176 (decompn.)	$C_8H_9ClN_4O_4S_2$	29.58	2.79	10.92	19.75	30.04	2.77	11.10	19.57
i-C_3H_7—	134 (decompn.)	$C_{12}H_{17}ClN_4O_4S_2$	37.84	4.50	9.31	16.84	37.68	4.54	9.38	16.70
$C_6H_5CH_2$—	158 (decompn.)	$C_{20}H_{17}ClN_4O_4S_2$	50.36	3.59	7.43	13.76	50.38	3.71	7.61	13.92
Cyclo-C_6H_{11}—	165 (decompn.)	$C_{18}H_{23}ClN_4O_4S_2$	46.89	5.47	7.69	13.91	47.13	5.67	7.81	13.98

EXPERIMENTAL

5-Chloro-2,4-bis-(alkylsulfamyl)anilines.—The required intermediates, 5-chloro-2,4-bis-(alkylsulfamyl)anilines (Table I), were prepared by treating an alcoholic or aqueous solution of 4 equivalents of the appropriate amine with crude 5-chloro-2,4-bis-(chlorosulfonyl)aniline prepared by the method of Novello (8). The products were recrystallized from alcohol.

2-Benzyl-7-benzylsulfamyl-6-chloro-1,2,3,4-benzothiatriazine 1,1-Dioxide.—A suspension of 7.8 Gm. of 5-chloro-2,4-bis-(benzylsulfamyl)aniline in 22 ml. of acetic acid and 1 ml. of water was treated with a solution of 1.2 Gm. of sodium nitrite in 9 ml. of sulfuric acid, keeping the temperature below 20°. The mixture was filtered and the filtrate was diluted with water. The gummy precipitate was washed with alcohol and recrystallized from acetone by addition of water, affording 5.5 Gm. of cream-colored crystals.

The other compounds (Table II) were prepared similarly. They showed positive diazo coupling tests with alkaline β-naphthol. Dumas nitrogen analyses were erratic.

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Communications

Synthetic Polymers as Potential Enteric and Sustained-Release Coatings

Sir:

We wish to report pharmaceutical applications of various copolymers as potential enteric and sustained-release coatings. These coatings are based on a series of partial esters (ethyl through decyl) of water-soluble copolymers of maleic anhydride with ethylenic compounds, *e.g.*, ethylene or vinyl methyl ether (1). Preliminary results indicate that films of these polymeric derivatives are brilliantly clear, chemically and physically stable, and nontoxic. Variations in ester chain length and degree of esterification effect a gradation in dissolution pH.

An increase in ester chain length for the essentially one-half ester produces a corresponding increase in dissolution pH. This highly significant property permits the design of pharmaceuticals which release their medicament only upon reaching a specific intestinal pH. Thus, specific coatings may be selected in order to obtain maximum therapeutic effect from those drugs which are absorbed in various areas of the intestinal tract. This effect on absorption by the gradation in dissolution pH is illustrated in Table I by the plasma salicylate levels from enteric coated acetylsalicylic acid tablets. Complete physiological availability (relative to a control) was obtained.

TABLE I.—PLASMA SALICYLATE LEVELS[a]

Coating Polymer	2 hr. Level, mcg./ml.	Peak Level, mcg./ml.	Peak Time,[b] hr.
Uncoated	38	38	2
1/2 Ethyl PVM/MA[c]	11	38	4
1/2 Isopropyl PVM/MA[c]	6	34	5
1/2 *n*-Butyl PVM/MA[c]	1	26	8

[a] Following administration of 652 mg. of acetylsalicylic acid to fasting patients. [b] Time of maximum plasma level. [c] Copolymer of vinyl methyl ether and maleic anhydride.

Sustained release may be obtained in two ways for those drugs which are well absorbed throughout the gastrointestinal tract. First, a series of polymer coatings dissolving at different pH may be used to coat small portions of the dose. Second, the rate of solubility of a coating based on a single polymeric ester can be decreased as desired by the addition of suitable extenders. In both instances, initial levels may be obtained by the administration of uncoated drug.

A comprehensive report on the characterization, coating techniques, and clinical evaluation will be forthcoming.

(1) Voss, A., and Dickhauser, E., U. S. pat. 2,047,398, July 14, 1936.

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Received May 5, 1962.
Accepted for publication June 14, 1962.

Book Notices

The Actinomycetes. Vol. 3. Antibiotics of Actinomycetes. By SELMAN A. WAKSMAN and HUBERT A. LECHEVALIER. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1962. viii + 430 pp. 17 × 25.5 cm. Price $18.

More than 400 substances and preparations isolated from cultures of actinomycetes are covered in this volume. More than 30 of these products have found practical application in chemotherapy. Part A covers the nature, formation, and activities of antibiotics; and Part B includes descriptions of the antibiotics produced by actinomycetes. A general index and an index of organisms are appended.

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Communications

Synthetic Polymers as Potential Enteric and Sustained-Release Coatings

Sir:

We wish to report pharmaceutical applications of various copolymers as potential enteric and sustained-release coatings. These coatings are based on a series of partial esters (ethyl through decyl) of water-soluble copolymers of maleic anhydride with ethylenic compounds, *e.g.*, ethylene or vinyl methyl ether (1). Preliminary results indicate that films of these polymeric derivatives are brilliantly clear, chemically and physically stable, and nontoxic. Variations in ester chain length and degree of esterification effect a gradation in dissolution pH.

An increase in ester chain length for the essentially one-half ester produces a corresponding increase in dissolution pH. This highly significant property permits the design of pharmaceuticals which release their medicament only upon reaching a specific intestinal pH. Thus, specific coatings may be selected in order to obtain maximum therapeutic effect from those drugs which are absorbed in various areas of the intestinal tract. This effect on absorption by the gradation in dissolution pH is illustrated in Table I by the plasma salicylate levels from enteric coated acetylsalicylic acid tablets. Complete physiological availability (relative to a control) was obtained.

TABLE I.—PLASMA SALICYLATE LEVELS[a]

Coating Polymer	2 hr. Level, mcg./ml.	Peak Level, mcg./ml.	Peak Time,[b] hr.
Uncoated	38	38	2
1/2 Ethyl PVM/MA[c]	11	38	4
1/2 Isopropyl PVM/MA[c]	6	34	5
1/2 *n*-Butyl PVM/MA[c]	1	26	8

[a] Following administration of 652 mg. of acetylsalicylic acid to fasting patients. [b] Time of maximum plasma level. [c] Copolymer of vinyl methyl ether and maleic anhydride.

Sustained release may be obtained in two ways for those drugs which are well absorbed throughout the gastrointestinal tract. First, a series of polymer coatings dissolving at different pH may be used to coat small portions of the dose. Second, the rate of solubility of a coating based on a single polymeric ester can be decreased as desired by the addition of suitable extenders. In both instances, initial levels may be obtained by the administration of uncoated drug.

A comprehensive report on the characterization, coating techniques, and clinical evaluation will be forthcoming.

(1) Voss, A., and Dickhauser, E., U. S. pat. 2,047,398, July 14, 1936.

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Year Book of Drug Therapy. 1961–1962 Series. Edited by HARRY BECKMAN. Year Book Medical Publishers, Inc., 200 East Illinois St., Chicago 11, Ill., 1962. 659 pp. 13 × 19.5 cm. Price $8.50.

Excellent reviews of published medical reports for the year ending September 1961 are given in the author's style. Dr. Beckman's own critical evaluation of the year's new drugs (blue pages) is interesting. Author and subject indexes are appended.

Punch-Card Methods in Research and Documentation. By MARTIN SCHEELE. Interscience Publishers, Inc. 250 Fifth Ave., New York 1, N. Y., 1961. xiv + 274 pp. 16 × 23 cm. Price $9.50.

This book, an English translation of a German text, covers terminology, technology, and methodology. It includes general rules and gives examples. Special emphasis is given to biology throughout the text. This is the second volume in a series on Library Science and Documentation. The first volume was "Tools for Machine Literature Searching."

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JOURNAL OF

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A publication of the American Pharmaceutical Association

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

September 1962 volume 51, number 9

Review Article

Prediction of Stability of Drugs and Pharmaceutical Preparations

By EDWARD R. GARRETT

TO PREDICT is "to tell or declare beforehand, foretell or prophesy" (190). In the realms of science, prediction is based on a knowledge of the relations among experimental values under observed experimental conditions. Prediction of the stability of drugs and pharmaceutical preparations depends on quantitative mathematical expressions that permit the calculation of rates of degradation by the substitution of the appropriate values for temperature, concentrations, pressure, time, pH, oxygen content, centrifugal or gravitational stress, light intensities and wavelengths, etc.

These quantitative relations can be obtained from the classical literature of chemistry and physics (83, 169). Judicious experimental design can permit the evaluation of the vital parameters with the minimum expenditure of time and labor. Thus, stability can be predicted for practical circumstances where study, *per se*, is experimentally or economically impractical or unsuitable.

The literature contains many articles on drug incompatibilities or instabilities for which references are cited (18, 134, 158, 192).

However, kinetic and predictive studies of drugs in the pharmaceutical literature are of relatively recent vintage. In fact, *ca.* 1950 can be considered the start of the modern era in the quantitative comprehension and prediction of drug stability.

Received from the College of Pharmacy, University of Florida, Gainesville.

This review will be concerned with those studies on drugs and pharmaceutical formulations where quantitative expressions were obtained to permit prediction of stability. It will consider the basic concepts and the rationales which can be applied to permit prognosis. This review will be practically limited to studies conducted in liquid media involving solvolytic processes and leave the aerobic, bacterial, enzymic, or photolytic degradations to another time or another reviewer. The prediction of stability of the complex systems characterized by colloidal suspensions, emulsions, ointments, and solid formulations will, in general, also be ignored.

The basic concepts of kinetics and their applications to the understanding of the mechanisms of reactions of many simple systems and reactive groups are given in many fine books on these subjects (2, 9, 14, 29, 43, 71, 72, 86, 103, 104, 123, 138). Their reading is recommended.

Predictive Use of the Apparent First-Order Rate Constant.—The chemical action of a solvent and other solutes on the drug in solution is of prime importance. A systematic approach to its understanding and quantification is to consider the rate of degradation of such a drug as proportional to the concentrations of the reactants in the rate-determining step, or proportional to the concentrations of the reactants in the equilibria which precede such a rate-determining step. The rate of drug transformation is given in concentration, c, change per unit of time; (time-unit^{-1}).

In most solvolytic reactions with constant conditions of temperature, pH, buffer kind and concentration, and ionic strength, the rate of change per unit of time is proportional to the first power of the concentration of the drug being transformed

$$dc/dt(M/\text{L./sec.}) = kc^1(\text{sec.}^{-1} \times \text{moles/L.}) \quad \text{(Eq. 1)}$$

The rate is considered as apparent first order since it varies as the first power of the concentration, c, of the substrate. On integration of Eq. 1, the logarithm of the concentration, c, when plotted against time is a straight line of slope $k/2.303$

$$\log c = -kt/2.303 + \log c_0 \quad \text{(Eq. 2)}$$

or the logarithm of the fraction of substrate untransformed when plotted against time is a straight line of the same slope

$$\log c/c_0 = -kt/2.303 \quad \text{(Eq. 3)}$$

where c_0 is the original concentration and k is in reciprocal time units (29, 71). It has been recommended that all rate constants be given in units of seconds for ease of literature comparison (161). Some typical semilogarithmic plots for the degradation of thiamine hydrochloride (47) are given in Fig. 1.

The rate constants, k, are frequently used to compare and estimate rates. An alternative constant to use is the half-life, $t_{1/2}$; the time when a concentration is reduced by half its initial value. For all apparent first-order reactions the $t_{1/2}$ is independent of the magnitude of the initial concentration of the substrate and is related to the first-order rate constant, k, by

$$t_{1/2}(\text{sec.}) = -2.303 \log 0.5/k = 0.693/k \quad \text{(Eq. 4)}$$

from the rearrangement of Eq. 3 where $c/c_0 = {}^1/_2$, $t = t_{1/2}$ and k is in sec.$^{-1}$.

Variations on this theme can be chosen to alculate the time of 5 or 10% loss of substrate, *i.e.*, $t_{0.95}$ or $t_{0.90}$, respectively (117). The half-life is only independent of initial substrate concentrations for first-order reactions. It has been used frequently to describe the stability of a drug and the rate dependencies (35, 95, 98, 130, 132, 193, 198). Nelson (143) provides a convenient table to determine the fractional loss of substrate for n half-lives. However for the purpose of a systematic presentation, the concept of the rate constant will be used in this paper as descriptive of the fraction of substrate remaining on exponential decay

$$c/c_0 = e^{-kt} \quad \text{(Eq. 5)}$$

It is apparent that if the apparent first-order rate constant, k, can be determined as a function of the physical conditions of the solution, the rate of degradation at a given concentration can be determined by substitution of that concentration, c, into Eq. 1; that the half-life under those conditions can be determined by substitution into Eq. 4; that the fraction of substrate remaining at any time, t, can be determined by use of Eq. 5.

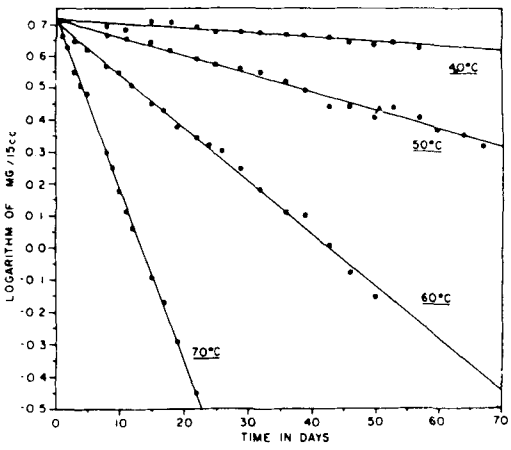


Fig. 1.—First-order plots of the thermal degradation of thiamine hydrochloride in a liquid multivitamin; logarithms of concentration (mg./15 ml.) against time in days. [Figure 1 of Garrett, E. R., This Journal, **45**, 470(1956) (47).]

To transform the half-life or first-order rate constants into other time units such as hours, days, years, etc., the conversions are

$$t_{1/2}(\text{time units}) = t_{1/2}(\text{in sec.})/(\text{seconds/time unit}) \quad \text{(Eq. 6)}$$

$$k(\text{time unit})^{-1} = k(\text{sec.}^{-1}) \times \text{sec./time unit} \quad \text{(Eq. 7)}$$

An appropriate procedure for studies that may be ambiguous is to verify the fact experimentally that the apparent first-order rate constant, k, is independent of substrate concentration. This has been done in many such cases (52, 55, 57, 66, 91, 114, 140, 154, 165, 193). Alternative methods of estimating apparent first-order rate constants are from the slopes of plots of concentration changes against time at the initial concentrations of substrate or by the Guggenheim method (81). Such procedures have been used with steroid hemiester hydrolysis and compared to the k values derived from classical plots (59, 61, 68).

Specific Acid-Base Catalyzed Solvolysis.—The simplest catalysts for solvolysis are hydrogen ions and hydroxyl ions so that the apparent first-order reaction rate constant k would be determined by

$$k = k_1[H^+] + k_6[OH^-] \quad \text{(Eq. 8)}$$

where catalysis by $[H^+]$ and $[OH^-]$ and the respective specific rate constants k_1 and k_6(L./*M*/sec.) are considered specific acid-base catalysis. In general, hydroxyl ion catalysis is negligible when a hydrogen ion catalyzed transformation is operative, and vice versa, so that one method of determining k_1 is to plot the observed rate constant, k, obtained from the slope of the plot of data according to Eq. 2, against the maintained hydrogen ion concentration.

As per Eq. 8 where the contribution of $k_6[OH^-]$ is negligible, the slope of such a plot estimates k_1 in L./*M*/sec. Obviously, k_6 may be similarly obtained by plotting k against $[OH^-]$ when the contribution of k_1 $[H^+]$ is negligible and k_6 in L./*M*/sec. can be estimated. A typical plot in this manner is given in Fig. 2 for the acid hydrolysis of psicofuranine (56). The negative intercept of the plot is due to the fact that the true molarity of hydrochloric acid is the apparent molarity lessened by the amount necessary to neutralize the psicofuranine.

Definition of Hydrogen and Hydroxyl Ion Concentrations and Activities.—The specific bimolecular rate constants are defined in terms of the ratio of the apparent first-order rate constant and the concentration or activity of the catalytic species. The specific rate constants of Eq. 8

$$k_1 = k/[H^+] \text{ when } k_6[OH^-] \sim 0 \quad \text{(Eq.8a)}$$

or

$$k_6 = k/[OH^-] \text{ when } k_1[H^+] \sim 0 \quad \text{(Eq. 8b)}$$

can be defined in terms of the stoichiometric strong acid or strong alkali concentration, *e.g.*, $[H^+]$ = [HCl] or $[OH^-]$ = [NaOH] on the postulate that the hydrogen or hydroxyl ion catalytic contributions are equivalent to the molarity of the strong acid or base. This is not necessarily true, however, as the effective hydrogen (or hydroxyl) ion concentration (activity) of a strong acid (or base) decreases from expectation with increased concentrations of the acid (or base) (89). The high activity of perchloric acid makes it one of the most ideal acids for the study of specific hydrogen ion catalysis (97, 98, 130–132). Ester hydrolysis is most generally catalyzed only by hydrogen and hydroxyl ions (specific acid-base catalysis) (9, 123) but consumes hydroxyl ions, so that if $k_6 = k/[OH^-]$ is to be determined, then the molar concentration of strong alkali should exceed the concentration substrate by at least tenfold, *e.g.*, $[OH^-] > 10c$ when $[OH^-]$ is taken as equivalent to [NaOH]. A specific example is the determination of $k/[OH^-]$ in the hydrolysis of acetylsalicylates (50). When the molar concentration of strong alkali approaches that of the substrate, bimolecular rate expressions must be used to calculate k_6 (9, 43, 104, 123). It is a similar situation for amides when hydrogen ions are taken up by the liberated amines the concentration of strong acid must greatly exceed the substrate concentration for pseudo first-order techniques to be used in the calculation of the apparent bimolecular rate constant k_1 (70).

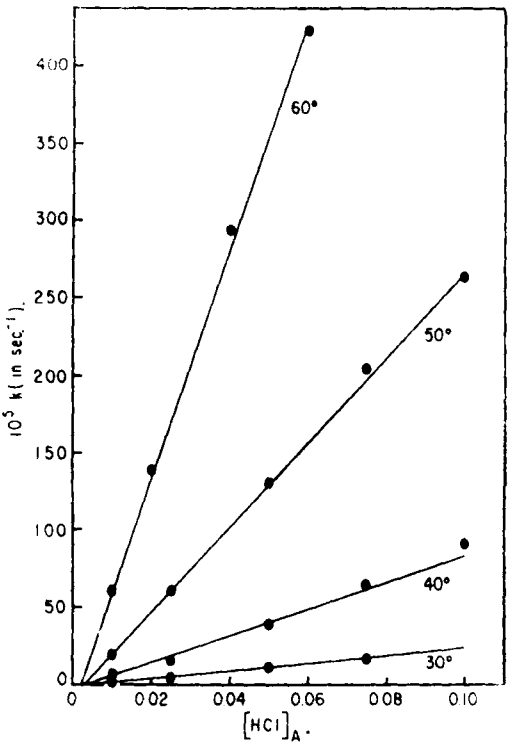


Fig. 2.—Rate constants, k in sec.$^{-1}$, for the apparent first-order degradation of psicofuranine as a function of apparent hydrochloric acid molarity. [Figure 3 of Garrett, E. R., *J. Am. Chem. Soc.*, **82**, 827(1960)(56).]

An alternative method is frequently used to calculate k_1 and k_6 where these values are defined in terms of the activities of hydrogen ions and hydroxyl ions, respectively.

The pH can be experimentally determined and, when k_6 $[OH] \sim 0$, the logarithmic transformation of Eq. 8 yields

$$\log k = \log k_1' - \text{pH} \quad \text{(Eq. 9)}$$

so that the slope of the logarithm of the apparent first-order rate constant against the experimentally determined pH is negative and equal to unity, and the antilog of the intercept yields the bimolecular rate constant k_1'. This k_1' value is related to the specific bimolecular rate constant k_1 by

$$k_1' = f_{H^+} k_1 \quad \text{(Eq. 10)}$$

where f_{H^+} is the activity coefficient (2, 89, 149) for the hydrogen ion and

$$k = k_1' \ 10^{-pH} \text{ when } k_6[OH^-] \sim 0 \quad \text{(Eq. 11)}$$

where the pH is the experimentally determined ($-\log a_{H^+}$), where a_{H^+} is the hydrogen ion activity.

A similar development for k_6' yields

$$\log k = \log k_6' - pOH = \log k_6 - pKw + pH \quad \text{(Eq. 12)}$$

The pK_w for a given temperature and solvent can be obtained from the literature (89, 149) so that k_6 can be calculated from the intercept of the plot of the logarithm of the apparent first-order rate constant against the experimentally determined pH. Thus, Eq. 8 is redefined in terms of pH as

$$k = k_1' \ 10^{-pH} + k_6' \ 10^{-(pKw - pH)} \quad \text{(Eq. 13)}$$

where the k_1' and k_6' values of Eq. 12 will frequently differ slightly from the k_1 and k_6 of Eq. 8 and yet be the desired values for prediction of solvolysis rates in intermediate pH ranges where pH is experimentally determined.

Comparisons of k_1 determined from stoichiometric catalytic concentrations and from pH measurements (*i.e.*, k_1') have been made in the cases of the solvolysis of the antibiotic streptovaricin (53) and the solvolysis of the antibiotic psicofuranine (56). An interesting comparison of measured pH and the pH calculated from kinetic rate constants is given for the solvolysis of acetylsalicylates (50).

It is frequently difficult to determine pH experimentally at high concentrations of hydrogen or hydroxyl ions so as to deduce the bimolecular rate constants k_1 and k_6 as from plots of Eqs. 9 and 12. However, stoichiometric acid concentrations can be converted to pH values corresponding to hydrogen ion and hydroxyl ion activities from the data on electrolytes in the literature (2, 89, 149).

For example, in concentrated hydrochloric acid solutions

$$pH = -\log f_{HCl}[HCl] \quad \text{(Eq. 14)}$$

where the mean activity coefficient of hydrochloric acid, f_{HCl}, is given at various temperatures, solvent mixtures, and HCl concentrations (89, 149). This definition of pH has been used in the study of the acid catalyzed solvolysis of hydrocortisone hemiester in alcohol-water mixtures (59) and of N-acetyl-*p*-aminophenol (114).

In the study of the acid catalyzed solvolysis of the antibiotic streptozotocin (57), the pH calculated from the activity coefficients (89) and the hydrochloric acid concentrations can be compared to the observed pH values of the solutions. Reasonable agreement is obtained above a pH of 1.2 and within 0.15 pH units below that value.

Similarly

$$pH = pKw - pOH = pKw + \log f_{NaOH}[NaOH] \quad \text{(Eq. 15)}$$

where f_{NaOH}, the mean activity coefficient of sodium hydroxide, and pKw are given in the literature for many solvent mixtures, temperatures, and solutes (89, 149). This definition of pOH has been used in the study of the alkaline hydrolysis of the antibiotics psicofuranine (56) and actinospectacin (69), where the k_6 values determined from the calculated hydroxyl ion activities and the stoichiometric [NaOH] have been compared.

Frequently, data exist in the literature to permit prediction of pH of buffer solutions under the conditions of preparation. An example of this procedure is in the study of barbital degradation in an ammonia buffer system (80) where the dissociation constants for the ammonia buffer system (8) and barbital (128) are given in the literature as a function of temperature. By the use of available activity coefficients (89, 115) and discreet approximations (7), the pH at various temperatures was calculated from the equation for the thermodynamic equilibrium constant for the buffer used. It is interesting to compare this approach (80) in the determination of the pH for the dependency of barbital kinetics to that of phenobarbital kinetics where the pH was determined experimentally (88).

Another method of characterizing hydrogen or hydroxyl ion concentrations is by the spectrophotometric determination of the degree of dissociation of a weakly acidic or basic indicator of known pKa where the spectrophotometric absorptivities of the charged and uncharged forms differ (73). The $[OH^-]$ calculated thusly has been used to determine the kinetic dependencies of the solvolysis of homatropine and atropine methyl-bromides (151).

Specific methods of calculating hydrogen and hydroxyl ion concentrations or activities for the prediction of the apparent first-order rate constants for the degradation of various pharmaceuticals are indicated in Table I. Methyl *dl*-α-phenyl-2-piperidylacetate (170) has exhibited specific acid-base catalyzed solvolysis where the log k *vs.* pH profile is given in Fig. 3 and the pH of minimum degradation is *ca.* 3. Specific acid-base catalyzed solvolysis has also been exhibited by the alkyl *dl*-α-(2-piperidyl)-phenyl-

acetates with pH *ca.* 3 of maximum stability (155) and N-acetyl-*p*-aminophenol with a pH 5–6 of maximum stability. The minimum of the catenary as exemplified in Fig. 3 and the pH of minimum overall rate at the isocatalytic point can be calculated from the specific acid-base catalytic constants as demonstrated in the literature (35, 113, 170). Many other examples of such catenaries are given in books on kinetics and mechanisms of reactions (2, 9, 43, 104, 123).

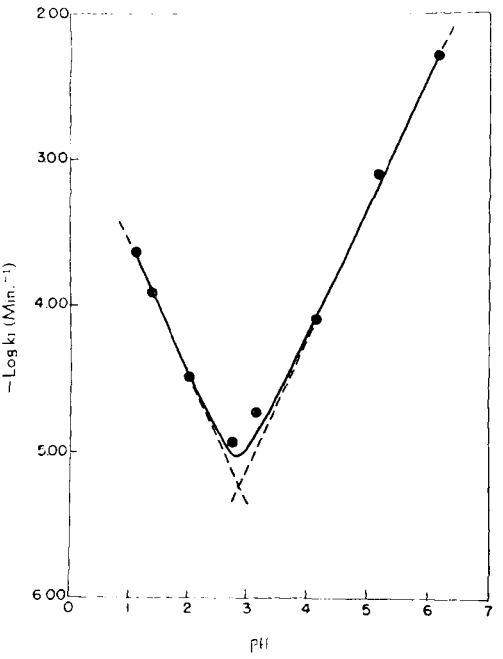


Fig. 3.—pH Dependency of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate at 80° [Figure 5 of Siegel, S., Lachman, L., and Malspeis, L., THIS JOURNAL, **49**, 431(1959) (170).]

Catalysis by Solvent.—If the apparent first-order rate constants calculated from Eqs. 8 or 13 do not agree with the observed values for intermediate pH ranges, then catalysis by solvent or solutes, intramolecular catalysis, or ionic strength must be considered as contributing to the overall rate.

The simplest modification of Eq. 8 is

$$k = k_1[H^+] + k_6[OH^-] + k_2 \quad \text{(Eq. 16)}$$

When the k plotted against $[H^+]$ has an intercept which cannot be attributed to hydroxyl ion attack, a solvent catalytic effect k_2 is implicated. This is further confirmed when k plotted against $[OH^-]$ has the same intercept or when

$$k = k_2, k_1[H^+] \sim 0 \sim k_6[OH^-] \quad \text{(Eq. 17)}$$

Such solvent attack on a degradable species is frequently apparent from the log k-pH profiles as with thiamine (193), pyridine-2-aldoxime methiodide (35), methyl pyrrolidylacetylsalicylate hydrochloride (51), and streptovaricin (53), which is given in Fig. 4.

General Acid-Base Catalysis.—Not only may hydrogen and hydroxyl ions and solvent alone catalyze the decomposition of drugs in solution, but charged species contributed from excipients or buffers may do so also. A general rule is that if solvent does catalyze, other catalytic species exist which can act as acids or bases with catalytic activity, and

TABLE I.—PREDICTIVE EQUATIONS FOR KINETICS OF SOLVOLYSIS OF DRUGS

Compound	Dependency of Apparent First-Order Rate Constant, k, in pH Region Studied[a]	Remarks	References
	Antibiotics		
Psicofuranine	$k_1 10^{-pH} f_{SH^+} + (k_4 10^{-pH} + k_6 10^{-pOH}) f_S + k_9 10^{-pOH'} f_{S^-}$	*b, d, g, h, i, j, k, l, q, s*	(56, 66)
Actinospectacin	$k_3 10^{-(pKw - pH)} f_{HS} + k_6 10^{-pOH} f_{S^-} + \Sigma_i k_i A_i^-$	*b, d, i, j, k*	(69)
Streptozotocin	$k_1 10^{-pH'} f_{SH^+} + (k_4 10^{-pH} + k_5 + k_6 10^{-(pKw - pH)}) f_S$	*b, d, g, h, i, l, m, s*	(57)
Streptovaricin	$k_1[H^+]^{(or\ k_1 10^{-pH})} + k_2$	*c, e, g, h, i, l, m, n*	(53)
Chloramphenicol	$k_2 + k_3 10^{-(pKw - pH)}$ (chloride solvolysis)	*b, d, i, s*	(91)
	$k_1[H^+]' + k_2 + k_3 10^{-(pKw - pH)} + \Sigma_i k_i \times HA_i + \Sigma_i B_i$ (amide solvolysis)	*b, d, g, h, i*	(97, 98)
	$k_1[H^+]' + k_2$ where $k_1 = k_1' 10^{m/D}[H_2O]$	*b, e, g* *t*	(132)
	Salicylates		
Acetylsalicylic acid	$k_1 10^{-pH} f_{HS} + (k_4 10^{-pH} + k_5 + k_6[OH^-]) f_{S^-}$ where $k_5 = k_5' + k_5''[C_2H_5OH]/[HOH]$	*d, e, f, g, h, i, j, k, l, m, q, t*	(34, 50, 58)
β-Cyclopentylpropionylsalicylic acid			
Trimethylacetylsalicylic acid		*t*	(50, 58)
Diethylacetylsalicylic acid			

TABLE I.—(*continued*)

Compound	Dependency of Apparent First-Order Rate Constant k, in pH Region Studied[a]	Remarks	References
Methyl pyrrolidylacetylsalicylate hydrochloride	$(k_2 + k_A^-[A^-] + k_3 10^{-(pKw - pH)}) f_{SH^+}$	*c, d, g, h*	(51)
	where $k_A^- = k_A' - 10^{(Z_A Z_B A \sqrt{\mu} + C\mu)}$	*u, v, x*	
	where $k_2 = k_2' 10^{Z_A Z_B A \sqrt{\mu}}$	*u, v*	
Diethylaminoethyl acetylsalicylate hydrochloride	$(k_1 10^{-pH} + k_2 + k_3 10^{-(pKw - pH)} + k_A^-[A^-] + k_{HA}[HA]) f_{SH^+}$ (overall rate)	*c, d, g, h*	
	where $k_A^- = k'_A{}^- \times 10^{(Z_A Z_B A \sqrt{\mu} + C\mu)}$	*u, v, x*	(52)
	$(k_1 10^{-pH} + k_2 + k_A^-[A^-] + k_{HA}[HA]) f_{SH^+}$ (to diethylaminoethyl salicylate hydrochloride)	*c, d, g, h*	
	$(k_3 10^{-(pKw - pH)} + k_A^- \times [A^-]) f^s{}_{H^+}$ (to acetylsalicylic acid)	*c, g, h*	
Diethylaminoethyl salicylate hydrochloride	$k_3 10^{-(pKw - pH)}$	*c, d, g, h, l, m, s*	(52)
Alkyl salicylates	$k_3[OH^-] f_{HS} + k_6[OH^-] f_S^-$	*b, d, e, f, k, q, s, t*	(154)
	where $k_3 = k_3' {}^{Z_A Z_B A \sqrt{\mu}}$	*u, v*	
Alkyl *m*- and *p*-hydroxybenzoates	$k_6[OH^-]$	*b, d, e, f, k, t*	(154)
	where $k_6 = k_6' 10^{Z_A Z_B A \sqrt{\mu}}$	*u, w*	
Acetylsalicylic acid anhydride	$k_2 + k_3 10^{-(pKw - pH)} + k_A^-[A^-] - k_{HA}[HA]$	*b, d, f, g, k, j, m, s*	(55)
	where $k_2 = k_2' 10^{(mID + b)}$	*t*	
	Steroids		
21-Hydrocortisone esters of hemisuccinate, hemiadipate, hemipimelate, hemisuberate, acetate, acrylate, hemi-α,α'-diethylglutarate, hemi-β,β'-dimethylglutarate, hemi-α,α'-diethylsuccinate	$k_6 10^{-(pKw - pH)}$	*c, e,*	(59)
Prednisolone hemi-β,β'-dimethylglutarate, 6α-methylprednisolone hemi-β,β'-dimethylglutarate	$k_6 10^{-(pKw - pH)}$	*b, d,*	(61, 68)
21-Hydrocortisone hemisuccinate	$k_1 10^{-pH'}$	*c, e, g*	(59)
	$k_6 10^{-(pKw - pH)}$	*c, e, j*	
	$k_1 10^{-pH} f_{HS} + (k_5 + k_6 10^{-(pKw - pH)}) f_S^-$	*c, e, h, i, j, l*	
Prednisolone	$k_3[OH^-]$	*c, d, k, p*	(84)
21-Hydrocortisone phosphate	$k_4 10^{-npH}$	*b, d, i*	(129)
	Alkaloids		
Atropine	$(k_1[H^+]' + k_3 10^{-(pKw - pH)}) \times f_{SH^+} + k_6 10^{-(pKw - pH)} f_S$	*b, d, g, i, j, k*	(113, 198)
Homatropine	$k_3 10^{-(pKw - pH)} f_{SH^+} + k_6 10^{-(pKw - pH)} f_S$	*b, d, i, j, k*	(150)
Homatropine methyl bromide, Atropine methyl bromide, Scopolamine, Acetylscopolamine, Aposcopolamine, Acetyltropate, Trimethylacetylscopolamine, Scopolamine methyl bromide, Trimethylacetyl scopolamine methyl bromide	$k_6[OH^-]'$	*b, d, i, k*	(151)
Acetylscopolamine methyl bromide, Aposcopolamine methyl bromide, Noratropine, Tropine phenylacetate, Tropine phenoxyacetate, Tropine *p*-nitrobenzoate	$k_6[OH^-]$	*b, d, k*	(49,67)
	$k_3 10^{-(pKw - pH)} f_{SH^+} + k_6 10^{-(pKw - pH)} f_S$	*b, d, i, j, k*	

TABLE I.—(*continued*)

Compound	Dependency of Apparent First-Order Rate Constant k, in pH Region Studied[a]	Remarks	References
Atropine ethylbromide Atropine benzyl chloride	$k_6[OH^-]'$	*b, d, j, k*	(152)
	Others		
Procaine	$(k_1[H^+]' + k_3 10^{-(pKw - pH)}) \times f_{SH^+} + k_6 10^{-(pKw - pH)} f_S$	*b, d, g, h, i, j, l, m, p, r*	(95, 130, 178)
Benzocaine	$k_1[H^+]'$	*b, d, g*	(130)
Procainamide	$k_1[H^+]'$	*b, d, g, p,*	(131)
Epinephrine	$k_1 10^{-npH}$ (racemization)	*b, d, g, p, s*	(165)
N-Acetyl-*p*-aminophenol	$k_1 10^{-pH'} + k_6 10^{-(pKw - pH)}$	*b, d, g, h, i, j, m, s*	(114)
2-(1-Naphthylmethyl)-2-imidazoline	$k_3 10^{-(pKw - pH)} f_{SH^+} + k_6 10^{-(pKw - pH)} f_S$	*b, d, i, j, k, q*	(171)
Alkyl *dl*-α-(2-piperidyl)-phenylacetates	$(k_1 10^{-pH} + k_3 10^{-(pKw - pH)}) \times f_{SH^+}$	*b, d, g, h, i, o*	(155, 170)
	where $k_1 = k_1' 10^{Z_A Z_B A \sqrt{\mu}}$	*g, u, w*	
	where $k_3 = k_3' 10^{Z_A Z_B A \sqrt{\mu}}$	*i, u, v*	
Pyridine 2-aldoxime methiodide	$(k_1 10^{-pH} + k_3 10^{-(pKw - pH)}) \times f_{SH^+}$	*b, d, g, h, i, j, k*	(35)
Chlorobutanol	$k_2 + k_3 10^{-(pKw - pH)}$	*b, d, g, h, i, l, m, r, s*	(140)
Barbital Phenobarbital	$(k_3 10^{-(pKw - pH)} + \Sigma_i k_i B_i) \times f_{HS} + (\Sigma_i k_i B_i + k_6 10^{-(pKw - pH)}) f_{S^-}$	*b, d, g, i, j, q*	(80, 88, 181)
p-Chlorobenzaldoxime	$k_1[H^+] - k_{-1}$ [Products]	*c, e, g*	(60)
N-Butylformamide	$k_1[H^+]$	*b, d, g*	(70)
Thiamine	$(k_1 10^{-pH''} + k_2 + k_3 10^{-(pKw - pH)} + \Sigma_i k_i A_i) f_{SH^{++}} + k_8 f_S$	*b, d, g, h, i, j, k, p, q, s*	(193)
	where $k_2 = k_2' 10^{Z_A Z_B A \sqrt{\mu}}$	*u, v*	
	where $k_3 = k_3' 10^{Z_A Z_B A \sqrt{\mu}}$	*u, v*	
	where $k_1 = k_1$, $k_8 = k_8$	*m*	
	where $k_8 = k_8$	*r*	

[a] SH^+ represents a protonated substrate in equilibrium with an uncharged substrate, S, with a dissociation constant K_a. The uncharged substrate, S, may lose a proton and may equilibrate with a negatively charged substrate, S^-. The symbol HS represents an acidic, uncharged substrate of a dissociation constant Ka, which may equilibrate with an anionic substrate, S^-. The symbol pH is for the experimentally determined value, $pH' = -\log f[HCl]$ and $pOH = -\log f[MOH]$ where f is the mean activity coefficient (2). The pH'' are values determined or calculated from the literature for the buffers used. Other symbols are $[H^+] = [HCl]$, $[H^+]' = [HClO_4]$, $[OH^-] = [NaOH]$. The $[OH^-]'$ is the hydroxyl ion concentration determined by an indicator method. The hydrogen or hydroxyl ion activity is frequently expressed in the form $a_{H^+} = 10^{-pH}$ or $a_{OH} = 10^{-pOH} = 10^{-(pKw - pH)}$. The general form of the kinetic expressions given is $(k_1[H^+] + k_2 + k_3[OH^-])f_{HS\ (or\ SH^+)} + (k_4[H^+] + k_5 + k_6[OH^-])f_{S^-\ (or\ S)} + (k_8 + k_9)[OH^-]f_{S^=\ (or\ S^-)}$ where $f_{HS\ (or\ SH^+)} = 1/(1 + Ka/[H^+])$ is the fraction of substrate as the acid form (uncharged or positively charged), where $f_{S^=\ (or\ S)} = 1/(1 + [H^+]/Ka_1)$ is the fraction of substrate as the base form (negatively charged or uncharged) and if another proton is removed, then $f_{S^-\ (or\ S^-)} = 1/(1 + Ka_2/[H^+])$ is the fraction of substrate further dissociated as a doubly or singly charged anion. The $[H^+]$ in these definitions of fractions of substrate is usually defined in terms of the measured hydrogen ion activity, *i.e.*, 10^{-pH}. The A_i^- and B_i represent charged and uncharged general bases respectively, whereas the HA_i and H^+B_i represent uncharged and charged general acids, respectively, so that $\Sigma_i k_i A_i^-$, $\Sigma_i k_i B_i$, $\Sigma_i k_i Ha_i$, and $\Sigma_i k_i H^+ B_i$ represent the contribution of solutes to general acid-base catalyzed solvolysis of the substrate. [b] Values at all operating temperatures can be calculated from $k_i = Ae^{-\Delta Ha/RT}$ or $\log k_i = \log A - \Delta Ha/2.303\ RT$ where A and ΔH_a can be derived from values given in the references. [c] Studied at one or two temperatures only. [d] Aqueous solutions. [e] Aqueous alcohols (methanol, ethanol, propylene glycol). [f] Aqueous dioxane or aqueous acetone. [g] Strong acid region. [h] Acetate buffer region. [i] Phosphate buffer region. [j] Mildly alkaline region. [k] Strong alkali region. [l] General acid-base catalysis checked and not observed with acetic acid-acetate buffers. [m] Ionic strength effects checked and not observed in region studied. [n] Oxidative degradation checked and not observed in region studied. [o] General acid-base catalysis checked and not observed with phthalic acid-phthalate buffers. [p] Oxidatively degraded when oxygen present. [q] Dissociation constants, Ka, at all operating temperatures can be calculated from Arrhenius expressions or other data. [r] General acid-base catalysis checked and not observed. [s] Checked at different substrate concentrations for first order with respect to substrate. [t] Rates studied as functions of dielectric constant where D is dielectric constant and m and b are constants. [u] $Z_A$and Z_B are the charges on the reacting ions and $\mu = \frac{1}{2}\Sigma c_i Z_i^2$ is the ionic strength. [v] $Z_A Z_B A$ is negative. [w] $Z_A Z_B A$ is positive. [x] The constant C is positive.

conversely. An example of this is the assignment of intramolecular catalysis to the pH-independent hydrolysis of the aspirin anion (50) rather than general acid-base catalysis by water since variations in acetic acid-acetate buffer concentrations had negligible effect on the hydrolysis (34).

Thus, Eq. 8 which has been extended to become Eq. (16) can be further modified

$$k = k_1[H^+] + k_6[OH^-] + k_2 + \Sigma_i k_i[HA_i] + \Sigma_i k_i[A_i^-] + \Sigma_i k_i[B_i] + \Sigma_i k_i[B_i H^+] \quad \text{(Eq. 18)}$$

where B is a general base and BH^+ its conjugate acid, where HA is a general acid and A^- is its conjugate base according to the treatment of Frost and Pearson (43).

An appropriate method to evaluate general acid-base effects is to determine the apparent first-order rate constant in the buffered vehicle and subtract those rate contributions which can be studied separately, such as k_1 $[H^+]$, k_6 $[OH^-]$, and k_2. The excess in degradation rate can be assigned to general acid-base catalysis provided

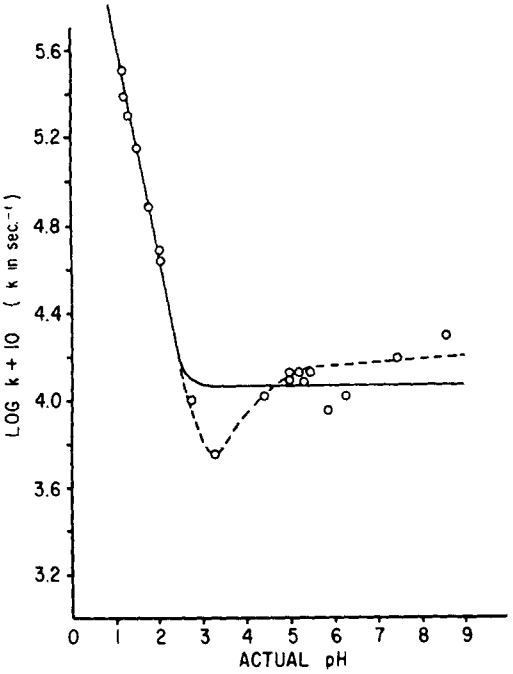


Fig. 4.—The pH profile of the degradation rates of streptovaricin C based on the 432 mμ absorbance disappearance in 25% ethanol at 30.3°. [Figure 3 of Garrett, E. R., THIS JOURNAL, **48**, 169(1959) (53).]

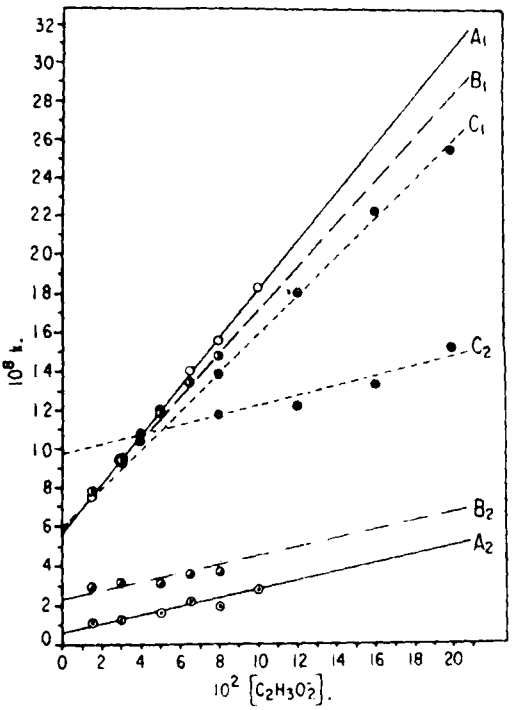
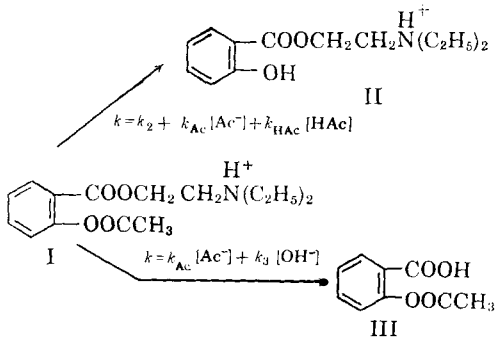


Fig. 5.—Effect of varying acetate ion concentration at constant ionic strength, μ, at several pH values on the simultaneous first-order rate constants of the hydrolyses to diethylaminoethyl salicylate hydrochloride, II, (curves A_1, B_1, C_1) and to aspirin, III, (curves A_2, B_2, C_2) of 10×10^{-4} *M* diethylaminoethyl acetylsalicylate hydrochloride, I, at 30.3°. ○, A, pH 4.00, 0.100 μ., ◑, B, pH 4.62, 0.100 μ., ●, C, pH 5.21, 0.200. [Figure 8 of Garrett, E. R., *J. Am. Chem. Soc.*, **80**, 4049 (1958) (52).]

that ionic strength effects have been controlled by varying buffer concentrations at constant pH and constant ionic strength. The use of a neutral salt such as sodium or potassium chloride is recommended.

A graphic example of a complex general acid-base catalyzed hydrolysis is given in Fig. 5 for the hydrolysis of diethylaminoethyl acetylsalicylate hydrochloride, I (52).



The apparent first-order rate constant, k, is plotted against the acetate ion concentration, $[Ac^-]$, at constant pH and constant ionic strength, μ. The curves A_1, B_1, and C_1, each at a different pH value, have the same intercept, k_2, and thus determine the solvent effect on the hydrolysis of I to diethylaminoethyl salicylic acid hydrochloride, II. The varying slopes, k_{Ac}', of lines A_1, B_1, and C_1 represent the rate constants for acetate ion and acetic acid catalyzed solvolysis of I to II. This k_{Ac}' can be dissected into the component contributions of base (k_{Ac}) and acid (k_{HAc}) catalytic rate constants since

$$k_{Ac}'[Ac^-] = \{k_{Ac} + k_{HAc}/K_a'[H^+]\}[Ac^-] \quad \text{(Eq. 19a)}$$

$$= k_{Ac}[Ac^-] + k_{HAc}[H^+][Ac^-]/Ka' \quad \text{(Eq. 19b)}$$

$$= k_{Ac}[Ac^-] + k_{HAc}[HAc] \quad \text{(Eq. 19c)}$$

where Ka′ is the dissociation constant of acetic acid.

Thus, from the slopes, k_{Ac}', of lines A_1 and A_2 in Fig. 5, the known $[H^+]$ of each curve and the relation

$$k_{Ac}' = k_{Ac} + (k_{HAc}/Ka')[H^+] \quad \text{(Eq. 20)}$$

the acid-base catalytic constants k_{Ac} and k_{HAc} were calculated (52).

The curves A_2, B_2, and C_2 for the hydrolysis of I to aspirin, III, have the same slope within experimental error, and thus only general base

catalysis is concluded. The intercepts of these lines vary and are consistent with hydroxyl ion catalysis.

Since most pH studies of pharmaceutical degradation rates are conducted in acetic acid-acetate, phosphate, borate, or similar buffers, the potential catalytic action of these species must be evaluated. Such effects have also been observed in the hydrolysis of phenylacetates (13, 23, 162) and in the solvolysis of aspirin anhydride (55). In the latter case, undissociated acetic acid had an inhibitory effect. General base catalysis has been shown for thiamine at pH values less than 7 (193), for the antibiotic streptozotocin by the monohydrogen phosphate anion (57), for methyl pyrrolidylacetylsalicylate hydrochloride by acetate ions (51), for barbital and phenobarbital by ammonia (80, 88, 181), for the antibiotic actinospectacin (69) by phosphate, carbonate, and borate buffers. General acid-base catalysis of chloramphenicol (97, 98) has been determined for monohydrogen phosphate ion, undissociated acetic acid, and both monohydrogen and the dihydrogen citrate ions. For the general case where general acid-base catalysis has been found and may be expected, the reader is referred to texts (9, 43, 123). General acid-base effects were tested for but not observed with acetate buffers for psicofuranine (56), for aspirin (34), the antibiotics streptovaricin (53) and streptozotocin (57), diethylaminoethyl salicylate hydrochloride (52), procaine (95, 130), and chlorobutanol (140). They were not observed with phosphate buffers for chlorobutanol (140), for phthalate buffers for alkyl *dl*-α-(2-piperidyl)-phenylacetates (155, 170), and in the high pH region for thiamine (193).

In actuality many of the mechanisms which follow the basic equations for general base catalysis actually go through a nucleophilic participation in the rate-determining step. Examples are given in Bender's fine review (12). Nucleophilic catalysis is involved in the solvolysis of anhydrides (32, 74–77, 79, 108–110) and also aspirin anhydride (55), in the imidazole (13), N-methylimidazole (24), and 4-methylpyridine (108) catalysis of *p*-nitrophenylacetate hydrolysis (24), and in the acetate ion catalyzed solvolysis of methyl pyrrolidylacetylsalicylate hydrochloride (51).

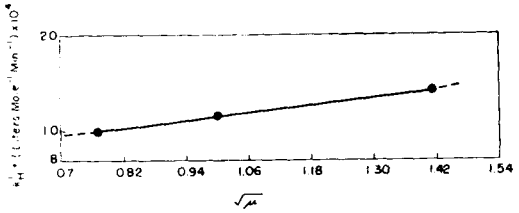


Fig. 6.—Influence of ionic strength on the velocity of a hydronium ion catalyzed solvolysis of a protonated substrate. [Figure 8 of Siegel, S., Lachman, L., and Malspeis, L., THIS JOURNAL, 48, 431(1959)(170).]

Ionic Strength and Dielectric Constant Effects.—The general expression that relates an observed bimolecular rate constant, k_i, with ionic strength, dielectric constant, and other thermodynamic properties of the solution of reactants for ion-ion reactions (1, 2) is

$$\log k_i = \log k_i' + z_A z_B A \sqrt{\mu}/(1 + \beta\sqrt{\mu}) \quad \text{(Eq. 21)}$$

so that

$$k_i = k_i' \, 10^{z_A z_B A \sqrt{\mu}/(1 + \beta\sqrt{\mu})} \quad \text{(Eq. 22)}$$

which to a first approximation may be given as

$$k_i = k_i' \, 10^{(z_A z_B A \sqrt{\mu} + C\mu)} \quad \text{(Eq. 23)}$$

or as

$$\log k_i = \log k_i' + z_A z_B A \sqrt{\mu} \quad \text{(Eq. 24)}$$

so that

$$k_i = k_i' \, 10^{z_A z_B A \sqrt{\mu}} \quad \text{(Eq. 25)}$$

where $\mu = {}^1/_2 \Sigma C_i z_i^2$, the ionic strength, z_A and z_B are the charges on the reacting species, and A and β are functions of the properties of the solution, *e.g.*, dielectric constants, temperature, etc., and thermodynamic constants and can be obtained from the literature (2, 89, 149).

Thus the dependence of log k_i *vs.* $\sqrt{\mu}$ should be a straight line of positive slope for the reactions of ions of like charge sign and negative slope for ions of unlike charge sign. An example of the application of Eq. 24 is given in Fig. 6 for the reaction of the positively charged hydronium ion with the positively charged ester, methyl *dl*-α-phenyl-2-piperidylacetate (170), where the plot of the logarithm of the specific hydrogen ion catalytic rate constant against the square root of the ionic strength is positive. When the positively charged ester is reacted with the negative hydroxyl ion, the salt effect is negative, as evidenced from Fig. 7 where such a plot has a negative slope (170). Such effects have been discussed in detail with many examples by Bell (9) and Amis (2). They have been observed in the acid and base catalyzed hydrolysis of carboxymethyltriethylammonium halide (10, 11, 145).

The salt effect is negative, *i.e.*, $z_A z_B A$, for the hydroxyl ion and general base, A^-, catalyzed solvolysis of the positively charged methyl pyrrolidylacetylsalicylate hydrochloride (51) and of the positively charged thiamine (193); for the

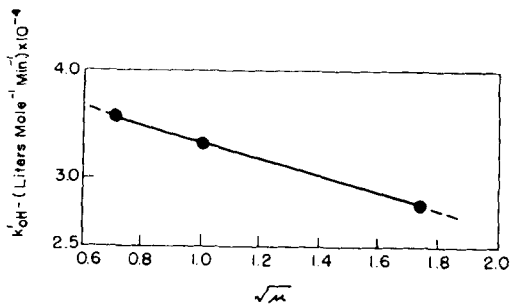


Fig. 7.—Influence of ionic strength on the velocity of a hydroxyl ion catalyzed solvolysis of a protonated substrate. [Figure 9 of Siegel, S., Lachman, L., and Malspeis, L., THIS JOURNAL, **48**, 431 (1959) (170).]

general base, A^-, catalyzed solvolysis of the protonated diethylaminoethylacetylsalicylate hydrochloride (52). The salt effect is positive, *i.e.*, $z_A z_B A$ for the hydroxyl ion catalyzed solvolysis of the negatively charged *m*- and *p*-hydroxybenzoates (154). In one study, salt effects were observed for the solvolysis of barbital (80) but were not quantified.

Salt effects were checked for but not observed in the pH regions studied for the acid and base catalyzed solvolysis of protonated procaine (95, 130), the solvolysis of N-acetyl-*p*-aminophenol (114), the acid solvolysis of chlorobutanol (140), the acid catalyzed solvolysis of doubly positively charged thiamine (193), the solvent catalyzed solvolysis of streptozotocin (57) and streptovaricin (53), the solvolysis of chloramphenicol in phosphate buffer (98), the hydroxyl ion catalyzed solvolysis of diethylaminoethyl salicylate hydrochloride (52), and the solvolysis of acetylsalicylic acid anhydride (55).

Also a plot of log k_i *vs.* the reciprocal of the dielectric constant at $\mu = 0$ should yield a straight line of negative slope for ions of like charge sign and of positive slope for ions of unlike charge sign (1, 2).

It follows that the use of miscible water-organic solvents where the dielectric constant decreases with more of the organic solvent should not increase the stability of a drug if the rate-determining step involves reactants of unlike charge. It can be predicted that the use of alcohol-water, propylene glycol-water, dimethylacetamide-water, sugar-water, or glycerol-water solutions will tend to increase the instability of protonated drugs subject to general or specific base catalysis in the presence of buffer anions if this is the major catalytic species. Examples of such predictions would be the hydrolysis of the scopolamine methylbromides (49, 67), atropine ethyl and benzyl bromides (152), the pH 3–7 solvolysis of methyl *dl*-α-phenyl-2-piperidylacetate (170), and alkyl *dl*-α-(2-piperidyl)-phenylacetates (155), where the major hydrolysis has been assigned to hydroxyl ion attack on a positively charged molecule. Certainly, in the case of the reaction of similarly charged species, such as in the hydroxyl ion catalyzed solvolysis of an anion (59, 61, 68) or the hydrogen ion catalyzed solvolysis of a cation (35, 95, 113, 130, 152, 155, 170, 198), the stability should be increased by decreasing the dielectric constant. The example of the decreased rate of fading of bromphenol blue (negatively charged quinoid dye ion) with hydroxyl ion when the alcohol content of water-ethanol and water-methanol solvents is increased (2, 3) is discussed in detail by Amis (1).

In the limiting case of the attack of an ion on a dipolar molecule (1, 2)

$$\log k' = \log k'_D + K \frac{z\mu}{D} \qquad \text{(Eq. 26)}$$

Thus, with an increase in dielectric constant, D, the rate of the reaction decreases for a positive ionic reactant and increases for a negative ion reactant. If the dielectric constant is decreased the rate should increase for positive ions and decrease for negative ions.

This equation and its indicated interdependencies has been applied with reasonable success to both the hydroxyl and hydrogen ion catalyzed hydrolysis of esters in water and organic solvent-water mixtures.

Examples are the water-ethyl alcohol and water-acetone alkaline solvolysis of ethylacetate (4, 156) and methyl propionate (157); the water-alcohol acid hydrolysis of aspirin (50), the water-acetone acid hydrolysis of methyl propionate (105), ethylacetate (141), ethyl formate (168), and ethyl dichloroacetate (142). In the case of the latter reactions of specific hydronium ion catalyzed ester hydrolysis where a preliminary equilibrium with water prior to the rate determining step may occur, the k' of Eq. 26 may be made specific with respect to water by dividing by the concentration of water. The relation of Eq. 26 is then demonstrated more validly (1, 105, 132, 141). A plot of data consistent to the requirements of this equation for the reaction between a cation and a dipolar molecule is given in Fig. 8 for chloramphenicol hydrolysis (132).

These relationships of rate with dielectric constant can also be used as methods to determine the nature of the reaction. In the hydrolysis of acylsalicylates (50, 58), the increase of rates of solvolysis of the aspirin anion with increasing ethanolic content along with the relative

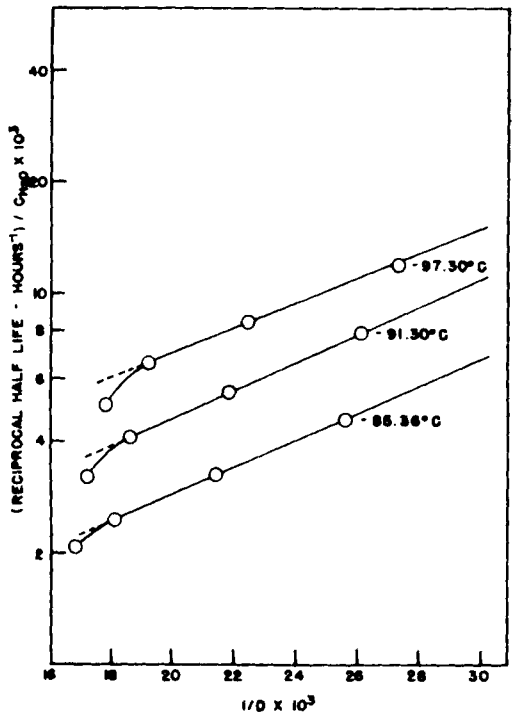


Fig. 8.—Influence of dielectric constant on reaction rates for the apparent reaction between the protonated chloramphenicol and the dipolar molecule water. [Figure 7 of Marcus, A. D., and Taraszka, A. J., THIS JOURNAL, **48**, 77(1959) (132).]

constancy of rate with increasing dioxane content, both concomitant with decreasing dielectric constant, did not implicate direct water attack on the aspirin anion. It led to kinetic evidence for intramolecular catalysis (50) and subsequently to the deduction of a possible rate-determining step in the attack of water or ethanol on an uncharged cyclic intermediate (58). Marcus (132) states that the rates for acid catalyzed solvolysis of protonated chloramphenicol in propylene glycol-water mixture does not fit the requirements for like charged ions and can be explained by the fact that the rate-determining step is an ion-dipolar molecule reaction between hydrogen ions and the hydrated amide. The log $k_1/[H_2O]$ values, when plotted against the reciprocal of the dielectric constant (84) as per Eq. 26, is linear and of positive slope. A point of Marcus (132) is well taken in that it is unwise to make any blanket assumption that lowering the dielectric constant (in general, by increasing the nonaqueous content of a solvent) will increase the stability of a drug. Predictions of stability as functions of the dielectric constant are valid on the basis of the operative mechanisms.

The limiting case for a dipolar molecule-dipolar molecule reaction is usually independent of ionic strength but dependent on dielectric constant (1, 2)

$$\log k_i = \log k_{(D=\infty)} - K(1/D) \quad \text{(Eq. 27)}$$

where a decrease in the dielectric tends to decrease the rate, and conversely.

There are many ways to relate the observed rate constant, k_i, to some function of the dielectric constant, D, for the interaction of dipolar molecules. A general expression to relate linearly the logarithm of the specific rate constant and some function of the dielectric ϕ_i (D), where m and b are constants, is

$$\log k_i = m_i\phi_i(D) + b_i \quad \text{(Eq. 28)}$$

Within small ranges of high dielectric constant, this relation is usually linear for practically any ϕ_i (D), *i.e.*, ϕ_1 (D) = nonaqueous solvent, ϕ_2 $(D) = D$, ϕ_3 $(D) = 1/D$, or ϕ_4 $(D) = (D - 1)/(2D + 1)$ (2, 72) as was shown in the case of the hydrolysis of aspirin anhydride (55). The latter expressions for ϕ_3 (*i.e.*, Eq. 27) and ϕ_4 can be extended to greater ranges of dielectric constant. Examples and further discussion can be found in the literature for the effect of solvent and dielectric constant on rates (38, 125, 153).

A plot of log k *vs.* $(D - 1)/(2D + 1)$ has been made for *t*-butyl chloride (124) in aqueous ethanol and in aqueous dioxane, acetone, ethanol, and methanol (38), for acetic anhydride (74, 78), benzoyl chloride (5, 78), benzyl (111), and substituted benzylsulfonates (112) in aqueous acetone, for ethylene bromo and iodohydrin (28) in aqueous ethanol, for *p*-nitrobenzylbromide (85) in aqueous dioxane. In most cases since m $(1/D) + b$ is the first part of the numerical expansion of $(D - 1)/(2D + 1)$, the fit of log k *vs.* $1/D$ is just as valid. Log k *vs.* log D appears to give the best possible linearity (38).

Solvolysis Rates, pH, and the Ionic Nature of the Substrate.—In many cases the hydrogen and hydroxyl ion catalyzed degradation rates of drugs in solution vary with the kind and amount of charge on the substrate molecule. The complex kinetic expressions that quantify these phenomena have been developed excellently by Higuchi (95) and Edwards (34). The approach of Edwards can be applied to the general case of the charged or uncharged acid substrate. The apparent dissociation constant, K_a, can be defined as

$$K_a = [S^-][H^+]/[HS] = [S][H^+]/[SH^+] \quad \text{(Eq. 28)}$$

In the solvolytic reactions of an uncharged acid the ionic form of the reacting substrate, the catalytic species and the specific rate constant can be given, respectively, as: HS, H^+, k_1;

HS, (H_2O), k_2; HS, OH^-, k_3; S^-, H^+, k_4; S^-, (H_2O), k_5; S^-, OH^-, k_6. With respect to k_2 and k_5 these values may or may not be dependent on the bimolecular attack of water or solvent on substrate and for this purpose are defined as first-order rate constants with respect to substrate rather than the alternatives, $k_2' = k_2/[H_2O]$, or $k_5' = k_5/[H_2O]$. Equivalent definitions and symbolisms and arguments can be constructed for the charged acid, SH^+.

It is reasonable to expect *a priori* that the specific bimolecular rate constant k_1 for the attack of a hydrogen ion on the undissociated uncharged acid, HS, would be less than the k_4 specific rate constant for such an attack on the oppositely charged anion, S^-. This does not necessarily imply that $k_1[H^+][HS] < k_4[H^+][S^-]$ so that the decomposition in acid solution is less than the decomposition in neutral solution since, to achieve a significant concentration of $[S^-]$, the concentration of $[H^+]$ must decrease below the dissociation constant, Ka. Similarly, although it is reasonable to expect *a priori* that the specific bimolecular rate constant k_3 for the attack of a hydroxyl ion on the undissociated uncharged acid, HS, would exceed the specific bimolecular rate constant for the attack of a hydroxyl ion on the anion S^-, it does not necessarily imply that decomposition in solutions of pH less than pKa exceeds that of solutions of pH greater than pKa. In the case of acetylsalicylates, the conditions defined by the dissociation constant of the acids where HS exists at very low concentrations of hydroxyl ion $[OH^-]$, indicate that $k_3[OH^-][HS]$ does not contribute significantly to the overall hydrolysis rate to products (34, 50).

The rate of decrease of the total concentration of substrate, $[HS]_T$, by solvolytic processes is equivalent to the sum of the rates of decrease of the undissociated acid [HS] and the anion $[S^-]$, which in turn can be defined by the specific rate constants

$$-d[HS]_T/dt = k[HS]_T \text{ (at constant pH)} = k([HS] + [S^-]) \quad \text{(Eq. 29)}$$

and

$$-d([HS] + [S^-])/dt = k_1[H^+][HS] + k_2[HS] + k_3[OH^-][HS] + k_4[H^+][S^-] + k_5[S^-] + k_6[OH^-][S^-] \quad \text{(Eq. 30)}$$

It has been shown (34, 95) that

$$k = (k_1[H^+] + k_2 + k_3[OH^-])/(1 + Ka/[H^+]) + (k_4[H^+] + k_5 + k_6[OH^-])/(1 + [H^+]/Ka) \quad \text{(Eq. 31)}$$

where

$$f_{HS} = 1/(1 + Ka/[H^+]) = [HS]/[HS]_T \quad \text{(Eq. 32)}$$

is the fraction of $[HS]_T$ as the undissociated acid [HS] and where

$$f_{S^-} = 1/(1 + H^+/[Ka]) = [S^-]/[HS]_T \quad \text{(Eq. 33)}$$

is the fraction of $[HS]_T$ as the dissociated acid $[S^-]$.

Analogous expressions could be derived for a singly charged (SH^+) or doubly charged substrate (SH^{++}).

If the substrate has two or more functionalities that vary the kind and amount of charge on the reacting molecule with pH, *i.e.*, pKa_1, pKa_2, etc., then a suitable variation of Eq. 31 could be developed on the same principles.

Frequently, kinetic ambiguity may exist. For example, a rate contribution may be assigned to $k_4[H^+][S^-]$ or to $k_2[HS] = k_4K_a[HS]$, as in the case of the hydrolysis of acetylsalicylate (50), and still be consistent with the kinetic data. Similarly, a rate contribution may be assigned to either $k_5[S^-]$ or $k_3[OH^-][HS] = k_5K_a[OH^-][HS]/Kw$, as in the case of the hydrolysis of hydrocortisone hemisuccinate (59).

Another alternative kinetic equivalence is $k_6'[OH^-]^2 f_{HS^{++}} = k_5 f_S$ at high pH values for the solvolysis of thiamine (193) where the mechanism of "spontaneous" decomposition was chosen on the basis of the fact that primary salt and general base catalytic effects could not be observed. Also, for thiamine solvolysis, where $k_3[OH^-]f_{HS^{++}} = k_5 f_{S^+}$, the interaction of hydroxyl ion and double protonated thiamine was chosen over the "spontaneous" decomposition of singly protonated thiamine since a negative dependence on ionic strength pointed to the reaction of a negative and positively charged ion (193). The hydroxyl ion catalyzed solvolysis of the uncharged alkylsalicylate, $k_3[OH^-]f_{HS}$, was chosen over its kinetic equivalent, the "spontaneous" hydrolysis of the ester ion, $k_5 f_{S^-}$, on the basis of the variation of rate with dielectric constant (154). Alternate choices for the solvolysis of pyridine 2-aldoxime methiodide on kinetic grounds are the specific base hydrolysis of the protonated substrate, $k_3[OH^-]f_{SH^+}$, or the spontaneous decomposition of the neutral substrate, $k_5 f_S$ (35).

Predictive equations for the kinetics of solvolysis of drugs are given in Table I in accordance with Eq. 31 for the dependency of the apparent first-order rate constant, k. Where the specific rate constants can be further defined in terms of salt effects (ionic strength) and dielectric constant, this has been done. The log k *vs.* pH profiles given in Figs. 3, 4, and 9–14 show interesting variations when the various specific bimolecular

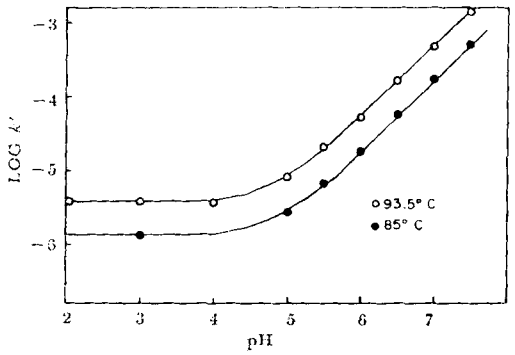


Fig. 9.—The log k-pH profile for the solvolysis of chlorbutanol. [Figure 9 of Nair, A. D., and Lach, J. L., THIS JOURNAL, **48**, 390(1959) (140).]

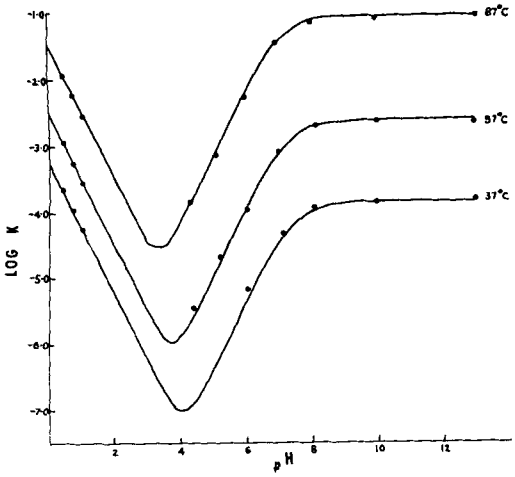


Fig. 10.—The log k-pH profile for the solvolysis of pyridine-2-aldoxime methiodide. [Figure 5 of Ellin, R. I., Carlese, J. S., and Kondritzer, A. A., THIS JOURNAL, **51**, 141(1962) (35).]

rate constants, k_i, $i = 1, 2 \ldots 6$ are observed as having significant contributions to the overall rate. Figure 3 for the protonated methyl *dl*-α-phenyl-2-piperidylacetate (170) exhibits the classic catenary when only specific acid-base catalysis is observed, as does the alkyl *dl*-α-(2-piperidyl)-phenylacetates (155) which exhibits a maximum stability at pH *ca.* 3. A similar profile for N-acetyl-*p*-aminophenol (114) has minimum at pH *ca.* 5–6. The profile of streptovaricin (53) Fig. 4, has a probable minimum plateau at pH values 3–4 due to a probable pH-independent solvolysis when the hydrogen ion concentration is lessened. Chlorobutanol (140), Fig. 9, exhibits no acid catalyzed solvolysis so that its maximum stability is a plateau at pH values less than 5. Pyridine 2-aldoxime methiodide (35), Fig. 10, exhibits minimal instability at pH *ca.* 3–4 and an interesting pH-independent plateau in the alkaline region due to a high sta-

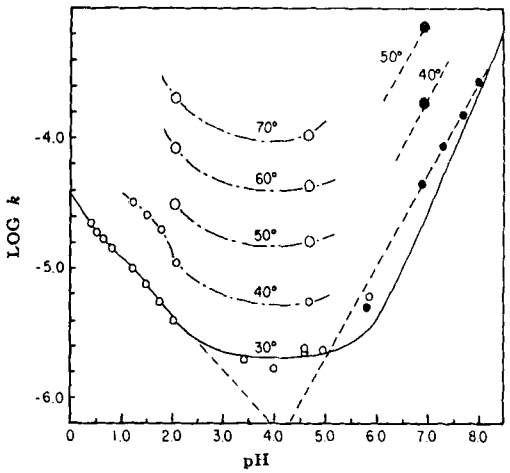


Fig. 11.—The log k-pH profile for the solvolysis of the antibiotic streptozotocin at 30°. [Figure 8 of Garrett E. R., THIS JOURNAL, **49**, 767(1960) (57).]

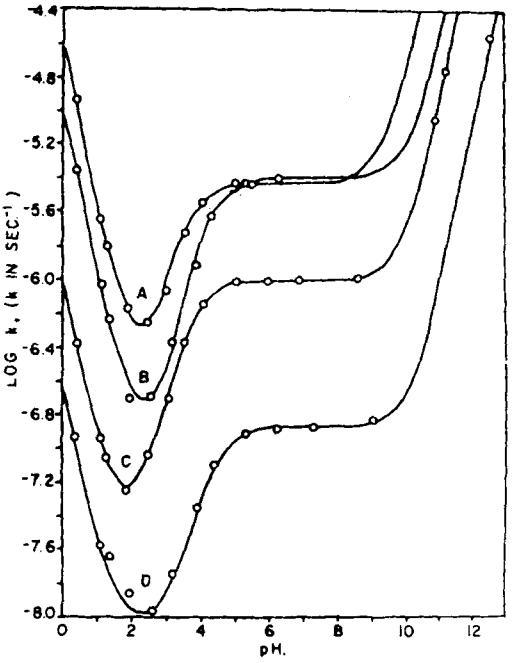


Fig. 12.—The log k-pH profile for the solvolyses of acylsalicylic acids, A, acetyl-; B,β-cyclopentyl-propionyl-; C, trimethylacetyl-; D, diethylacetyl-, at 25°. [Figure 1 of Garrett, E. R., *J. Am. Chem. Soc.*, **79**, 3401(1957) (50).]

bility of the oximate to hydroxyl ion attack. Streptozotocin (57) exhibits two deviations from the expected catenary in its log k-pH profile, Fig. 11. In the alkaline branch, rates in phosphate buffer exceed the specific base catalysis expected due to a general base catalysis by phosphate anions. On the other side of the arte minimum at pH *ca.* 4 in the acid branch, the log k *vs.* pH plots deviates from a slope of unity. Such kinetic data permit the calculation of a dis-

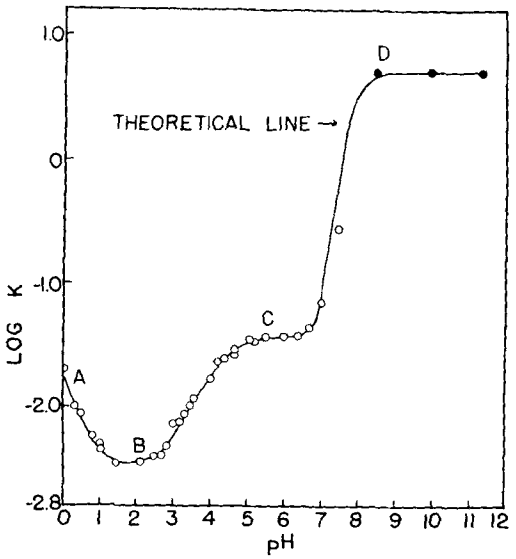


Fig. 13.—The log k-pH profile for thiamine at 96.4°. [Figure 11 of Windheuser, J. J., and Higuchi, T., This Journal, **51**, 354(1962) (193).]

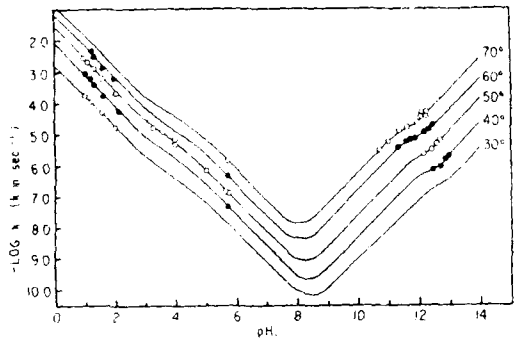


Fig. 14.—The log k-pH profile for psicofuranine. [Figure 4 of Garrett, E. R., *J. Am. Chem. Soc.*, **82**, 827(1960) (56).]

sociation constant difficultly obtainable by potentiometry, and the method described in the original paper (57) may be of interest. The hydrolyses of acetylsalicylic acids (34, 50) have an interesting log k-pH profile, Fig. 12, due to a pH-independent rate of anion solvolysis before hydroxyl ion catalyzed hydrolysis of the anion becomes significant. The pH of maximal stability of these compounds is *ca.* 3. Thiamine (193) also has an interesting profile, Fig. 13, and exhibits the extraordinary phenomenon of a large rate elevation within a short pH range that maintains constancy with large increases in pH. This phenomenon can be assigned to the change in the ionic nature of the substrate with pH where the new solution species of thiamine has a high but constant rate of solvolysis. Psicofuranine's (56) log k-pH profile, Fig. 14, exhibits the classical case of a substrate that can exist in three ionic forms in which the cationic form shows hydrogen ion catalyzed solvolysis, the anionic form hydroxyl ion catalyzed solvolysis, the neutral form is subject to both catalyses, and the pH of minimum hydrolysis is *ca.* 8–8.5.

The importance of a rate-pH profile cannot be underestimated for the prediction of the pH of minimum hydrolysis, the optimum for pharmaceutical stabilities. It can readily be seen that relationships among the magnitudes of the determined k_i values determine the pH of this minimum. Other pH minimum values so obtained are *ca.* pH 1–3 for thiamine (193), *ca.* pH 3 for hydrocortisone hemisuccinate (59) [which rate-pH profile resembles acetylsalicylic acid (34, 50) except for the lack of a significant k_4], and pH < 3 for aspirin anhydride (55) which has no specific acid catalyzed solvolysis similar to chlorobutanol (140). Actinospectacin (69) also showed no pharmaceutically significant acid instability and the barbitals (80, 88, 181) were not attacked by acid.

The isocatalytic point has been discussed for other solvolyses (107), ethylaminoacetate (19), and acetylcholine (25).

Approximative Methods for Log k-pH Relations.—As has been discussed, there are many instances where the ionic strength, general acid-base catalysis, or changes in the ionic nature of the substrate due to pKa properties, known or unknown, will give apparent nonlinear relations between log k and pH. In many cases the relation can be mathematically characterized, however, by an equation of the form

$$\log k = -n\text{pH} + \log k_i \qquad \text{(Eq. 34)}$$

or

$$k = k_i\, 10^{-n\text{pH}} \qquad \text{(Eq. 35)}$$

Similarly

$$k = k_i\, 10^{n\text{pOH}}$$

where the pH or pOH range of effectiveness must be defined. Such relations have been used to characterize the solvolysis of hydrocortisone phosphate in the neutral pH range (132), the racemization of epinephrine (165), the basic solvolytic degradation of actinospectacin (69), and the solvolysis of streptozotocin in phosphate buffers (57) where general base catalysis perturbed the alkaline branch of the specific acid-base catalytic catenary.

Frequently, such perturbations can also be accounted for by defining a rate constant for a particular hydrogen ion or hydroxyl ion range as

$$k = k'[OH^-] + k''; \; a < [OH^-] < b \quad \text{(Eq. 36)}$$

or

$$k = k'[H^+] + k''; \; a' < [H^+] < b' \quad \text{(Eq. 37)}$$

Examples and comparisons of the above two sets (Eqs. 34–37) of approximations with the values given by a true mechanistic dependency are given for actinospectacin (69) and psicofuranine (56, 66).

Prediction of Solvolytic Rates at Various Temperatures.—The quantitative relation of specific reaction rates and temperature is the Arrhenius expression (2, 9, 14, 29, 43, 71, 72, 86, 103, 104, 123, 138)

$$k_i = Ae^{-\Delta H_a/RT} \quad \text{(Eq. 38)}$$

where k is the specific rate of degradation or rate constant, T is the absolute temperature (°C. + 273), R is the gas constant (1.987 calories degree^{-1}mole^{-1}), A is a constant associated with the entropy of the reaction and/or collision factors, and ΔH_a is defined as the heat of activation.

The logarithmic form of Eq. 38

$$\log k_i = -(\Delta H_a/2.303R\,(1/T) + \log A \quad \text{(Eq. 39a)}$$

$$= -S/T + P \quad \text{(Eq. 39b)}$$

shows that the slope, $-S$, and intercept, P, of the plot log k *vs.* $1/T$ and, thus, ΔH_a and A, can be evaluated (Fig. 15) from several studies at various temperatures. Thus, the specific rate constants for any temperature can be calculated from a knowledge of these constants and applied to the prediction of the overall apparent first-order rate constant as previously discussed. It is also apparent that a rate constant at any temperature can be calculated from the knowledge of the heat of activation, ΔH_a and one rate constant at a known temperature (193). The footnotes of Table I specify if A and ΔH_a are available for the various solvolytic degradations of those pharmaceutically important compounds and a list of some of them is tabulated elsewhere (170). Additional sources of such values are in the texts previously cited and may be found for many others, as well as the kinetic dependencies of rates, in two fine compilations published by the National Bureau of Standards (173).

The effect of ΔH_a of the per cent of the organic component in mixed aqueous-organic solvents in the hydrolysis of esters is given by Tommila, *et al.* (154, 182–184). Graphs of this dependency generally pass through a minimum for alkaline solvolysis.

Higuchi and Busse (92) have pointed out that knowledge of the heats of activation for the

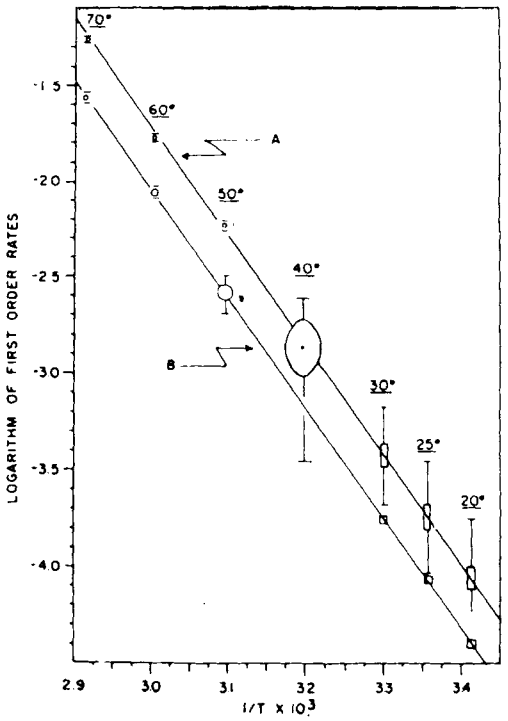


Fig. 15.—Arrhenius plots for thiamine hydrochloride in liquid multivitamin preparations. The circles represent the standard deviation of an experimental log rate in days and the rectangles the standard deviation of a predicted log rate. The horizontal lines represent the 95% confidence limits of experimental and predicted log rates. [Figure 3 of Garrett, E. R., THIS JOURNAL, **45**, 470(1956) (47).]

stability of drugs and the sterilization of microorganisms and their relative magnitudes can permit the proper choice of sterilizing times and temperatures for minimum loss of the drug. For procaine solutions, autoclaving at 120° is preferable to prolonged sterilization at 100°.

Comprehension of the significance of the knowledge of heats of activation can permit the prediction of the feasibility and optimum temperatures and time for the selective degradation of components in a mixture of drugs for the salvaging of lots contaminated by an unwanted material difficult to remove by other methods. This technique has been applied to a mixture of penicillins (65).

A special point must be made of the care that must be taken in estimating the heats of activation which are frequently deduced as "apparent" heats of activation (95, 140, 198).

If rate is dependent on hydroxyl ion concentration, $k_6[OH^-]$, and is determined at constant pH for several temperatures then

$$k_6[OH^-] = k_6Kw/[H^+] = k_6'Kw = Ae^{-\Delta Ha/RT} = A_{k_6'}e^{-\Delta H_{k_6'}/RT}\,A_{Kw}e^{-\Delta H_{Kw}/RT} = Ae^{-(\Delta H_{k_6'}+\Delta H_{Kw})/RT} \quad \text{(Eq. 40)}$$

and the heat of ionization of water, ΔH_{Kw}, must be subtracted to obtain the heat of activation of the specific hydroxyl ion catalyzed reaction $\Delta H_{k_0'}$.

Prediction of Drug Solvolysis in Heterogeneous Systems.—Since rate is a function of the concentration of the substrate, an apparent first-order reaction will become zero order with respect to substrate in saturated solutions as

$$\text{amount/volume/time} = kc_s = k_0c^0 = k_0 \quad \text{(Eq. 41)}$$

since c_s is the concentration of the substrate in the saturated solution.

It is thus possible to decrease the overall rate of hydrolysis of a substrate by solvolytic mechanisms by decreasing its solubility in the pharmaceutical vehicle. Examples are penicillin G procaine suspensions (172) and saturated solutions of acetylsalicylic acids (15, 48). Although the stability of acetylsalicylic acids in homogeneous solution (50) was in the decreasing order; diethylacetyl-, trimethylacetyl-, β-cyclopentylpropionyl-, acetyl-, in saturated solution, β-cyclopentylpropionyl-salicylic acid had the greatest stability due to its lowest solubility. When the kinetic dependencies of the apparent first-order rate constant, k, are known and the solubility, c_s, is also known as a function of pH and temperature, the stability of saturated solutions can be predicted.

The expression (48, 94, 133)

$$\text{pKa}' = \text{pH} + \log c_i/(c_s - c_i) \quad \text{(Eq. 42)}$$

may be used to predict the solubility, c_s, of a material of pKa′ at any given pH where c_i is the intrinsic solubility, *i.e.*, the solubility of the uncharged species which is presumed to be the determining factor of total solubility. The equation has been used to determine the solubility of sulfadiazine sodium (106) and secobarbital (185) in several solvent systems. For example, the stability of saturated solutions of barbitals could be calculated for any pH from the data on solubility (94) and the data on homogeneous kinetics (80, 88, 181) given in the literature.

In general, solubility also follows the Arrhenius law of Eq. 38 where ΔH_a represents the heat of solution, so that with the parameters of this equation, solubility c_s can be predicted for any temperature (54, 185). The complex system of saturated solutions of aspirin anhydride degrading to aspirin to salicylic acid can be quantitatively predicted for any temperature or pH (54). It was also shown, analogous to Eq. 40, that the apparent heat of activation for the degradation of saturated solutions of aspirin anhydride is actually the sum of the heats of solution and of activation of the homogeneous solution hydrolysis for aspirin anhydride (54).

Riegelman (159) has stated that in the heterogeneous system produced by surfactants with benzocaine that hydrolysis rates are more dependent on surfactant concentrations than the chain length of the polymeric surfactant. Anionic and cationic surfactants appear to stabilize the drug to base catalysis.

Bonus Values from Predictive Techniques.—Not only is information obtained from these studies that would permit the prediction of stability with varied substrate concentrations, pH values, salt concentrations, solvent changes, temperature variations, or saturated solutions, but additional information can be gleaned from judiciously designed experiments. The antibiotic psicofuranine is solvolytically degraded to adenine which is uniquely antagonistic to the antibacterial properties of its precursor (66). This was determined from concomitant biological and chemical assays of degrading solutions and the fact that a plot of biological assay *vs.* chemical assay was linear, but of negative intercept. Further investigation proved that the product adenine lessened the biological response. A decision was made that the excellent anticholinergic properties of acetylscopolamine methyl bromide were unique to this compound and not due to its ready gastrointestinal hydrolysis to the already established anticholinergic scopolamine methyl bromide, and that an expensive clinical trial of the former compound was justified (49). Comparison and understanding of the kinetics of solvolysis and solubilities of aspirin anhydride (54, 55) and aspirin (34, 48, 50), plus a comprehension of the rationales for aspirin absorption, led to the clinically confirmed prediction that aspirin anhydride would give higher blood levels of aspirin and less levels of salicylic acid than aspirin on oral administration to fasting subjects (54). The recommended conditions for autoclaving procaine solutions for minimum instability has already been cited (92). Stability studies were consistent with the fact that oral inefficacy of *p*-chlorobenzaldoxime (60) could be ascribed to gastric solvolysis but excluded this as an explanation for the oral inefficacy of N-butylformamide (70). The knowledge of the log k-pH profile of aspirin solvolysis (34, 50) suggested that a protonated amine in the molecule would permit a soluble, stable salicylic acid derivative to be formulated in an acid aqueous media which might readily hydrolyze to aspirin at the pH of the intestine.

TABLE II.—REPRESENTATIVE LOG k *vs.* pH PROFILES FOR VARIOUS KINETIC DEPENDENCIES OF THE APPARENT FIRST-ORDER RATE CONSTANT, k

Figure	k[a]	Substrate HS or S	Reference
3	$k_1[H^+] + k_3[OH^-]$	Methyl *dl*-α-phenyl-2-piperidylacetate	(170)
4	$k_1[H^+] + k_2$	Streptovaricin	(53)
9	$k_2 + k_3[OH^-]$	Chlorobutanol	(140)
10	$(k_1[H^+] + k_3[OH^-])f_{SH^+}$	Pyridine 2-aldoxime methiodide	(35)
11	$k_1[H^+]f_{SH^+} + (k_4[H^+] + k_6[OH^-])f_S$	Streptozotocin	(57)
12	$k_1[H^+]f_{HS} + (k_4[H^+] + k_5 + k_6[OH^-])f_{S^-}$	Acylsalicylic acids	(50)
13	$(k_1[H^+] + k_2 + k_3[OH^-])f_{SH^{++}} + k_8f_S$	Thiamine	(193)
14	$k_1[H^+]f_{SH^+} + (k_4[H^+] + k_6[OH^-])f_S + k_9[OH^-]f_{S^-}$	Psicofuranine	(56)

[a] f_S, f_{SH^+}, f_{HS}, or f_{S^-} represent the fraction of substrate, S or HS, in charged or uncharged form.

One of two such compounds did so readily hydrolyze to aspirin in slightly alkaline media, but both compounds unfortunately exhibited a high degree of general base catalyzed hydrolysis even by water alone in acidic solutions (51, 52). This did lead to a fundamental contribution since general base catalyzed hydrolysis of esters had been presumed to be nonexistent (9, 123) prior to the time of submission of the first paper for publication (51).

An intriguing stabilization technique has been introduced by Higuchi, Lachman, and co-workers (20, 96, 120–122). This technique was based on their comprehension of the fact that a complexed substrate might have negligible degradation rates in contrast to the uncomplexed substrate. Thus, knowledge of the equilibrium constants for the formation of such complexes and kinetic studies should permit the prediction of stability of complex-stabilized substrates. Examples are the stabilization of benzocaine (96, 116), procaine (122), tetracaine (121), and *p*-hydroxybenzoic acid (20) by caffeine, homologs (20, 120), and other agents (116). In many of these instances, the complexed substrate did not hydrolyze at all and the appearance of degradation products could be ascribed solely to the free substrate in equilibrium with the complex. A more recent paper (160) substantiates this principle by demonstrating that boric acid chelation of the catechol nucleus of epinephrine stabilizes against degradation by the attack of sulfite and bisulfite ions on the optically active side chain (100, 102, 118). It has also been shown that lithium chelates with alkylsalicylates and inhibits hydroxyl ion attack (154). This thesis has led workers to consciously seek to find if complexation exists and inhibits degradation rates. Unfortunately, success has not met all such attempts (155, 193).

A more detailed review of the literature on complexation of pharmaceuticals and its potential applications, including the stabilization of these pharmaceuticals, has been recently published by Walker (186, 187). The role of chelation in drug stabilization and other phenomena is covered in a review by Foye (42).

Frequently it is felt that modification of a pharmaceutical compound by appropriate substituents, if therapeutic efficacy was not decreased, could increase the stability of the pharmaceutical. It is apparent that steric blocking was effective in the case where the size and complexity of the acid groups in acetylsalicylic acids (50) and 21-steroid hemiesters (59, 68) varied. The Newman "rule of six" (127, 144) has great predictive power in the estimation of steric hindrance of ester solvolysis.

Another powerful predictive tool is the Hammett linear free energy relation of sigma-rho (43, 86) where

$$\log k = \log k_0 + \sigma\rho \qquad \text{(Eq. 43)}$$

where the substitutent constant σ is determined by the nature of the substituent and independent of the reaction; where the reaction constant ρ is a constant for all substituents. By definition, $\sigma = 0$ when $k = k_0$, the rate constant of the unsubstituted compounds. Hammett determined σ values for various substituents of benzoic acid as the logarithm of the ratio of the dissociation constants of the *m*- and *p*-substituted benzoic to unsubstituted benzoic acids. Astonishing linearity of log k *vs.* σ plots exists for many reactions. Taft (174–176) has extended this concept to the reactions of aliphatic compounds and *ortho*-substituted benzene derivatives. The contributions of Hammond (87) and Grunwald and Winstein (82) should be noted. Recently, Portoghese and Malspeis (155) have shown that when the log k/k_0 for the log ratio of specific base catalyzed hydrolysis rate constant of some alkyl esters of *dl*-α-(2-piperidyl)-phenylacetic acids to that constant for the methyl ester is plotted against a σ obtained from a log ratio of specific base catalyzed hydrolysis of alkyl acetates, astonishing linearity is obtained (Fig. 16). These

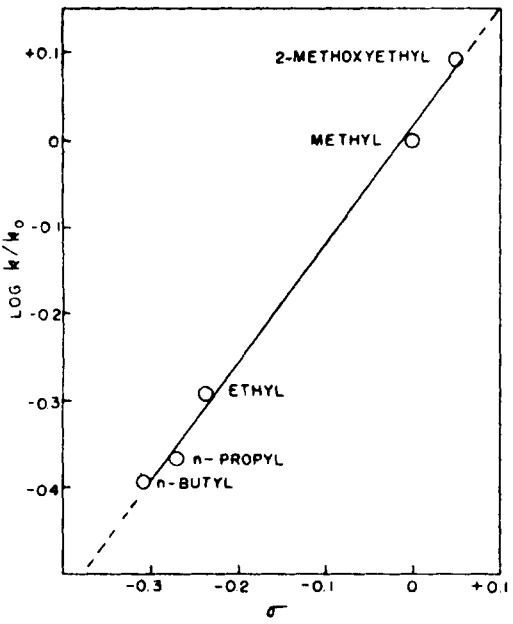


Fig. 16.—Correlation of log k/k_0 values for the specific base-catalyzed hydrolysis of alkyl esters of *dl*-α-(2-piperidyl)-phenylacetic acid at 80°, pH 5.98 with σ values of alkylacetates. [Figure 5 of Portoghese, P., and Malspeis, L., THIS JOURNAL, **50**, 494(1961)(155).]

facts imply that the σ and ρ constants available in the literature may permit the prediction of appropriate substituents to obtain the desired stability of many compounds, or that with a minimum of study on some substituent groups, the effects of many on stability can be quantitatively predicted.

It has also been shown that vitamin A esters of acids in anhydrous solvents have increased stability to proton attack with increasing Ka of such acids and yet in the absence of mineral acids, the instability of the vitamin A ester increases with the Ka of the ester acid portion (41).

Prediction of Stability of Drugs of Unknown Structure.—There is a need in pharmacy to study the changes in structures of molecules of unknown structure to predict stability. The stability of materials must be known for the establishment of treatment of assay standards and for the preparation of samples to be submitted for clinical evaluation.

The application, efficacy, and toxicity of an antibiotic or antibiotic complex is frequently well known before the components can be separated, purified, individually characterized, and structures assigned.

The stability of materials must be known for the establishment of treatment of assay standards and for the preparation of samples to be submitted for clinical evaluation.

Prediction of stability and design of formulations may be based on studies of the rates of change of some physicochemical characteristic. Correlation of changes in the physicochemical properties with the changes in the bioassays may serve as a valid argument for the use of the former. An obvious disadvantage is that variation in the latter may not necessarily reflect in the former although the converse is not probable.

Obvious advantages are that such physicochemical studies will be easy to conduct, less expensive, can lead to prediction of the conditions that affect degradation, and provide information on the properties of the intermediates and products.

Examples of the application of such a philosophy were the stability studies on the antibiotic fumagillin (33, 45, 64), an amebacide, which showed the necessity of protecting the material from light and oxygen and gave the rates of degradation in solution and crystal, as well as Arrhenius' expressions for predicting temperature effects. The ultraviolet absorptivities were correlated with biological activites (64). The prediction of the stabilities of the antitubercular streptovaricins (53) and the antibiotics, streptozotocin (57) and actinospectacin (69), have been previously discussed. The studies on the streptovaricins (53) also provided a correlation of biological activities with spectrophotometric absorptivities, the fact of increased stability in ethanol, physicochemical properties of the degradation product which would abet purification, and a differential kinetic method to assay for individual streptovaricins in the antibotic complex.

The stability studies on streptozotocin (57) correlated the polarographic and color assay due to an N-nitrosomethyl urea function with biological activity, kinetically observed a low pKa, and predicted that the high instability at the pH values of the blood would not permit significant blood levels of the antibotic to be observed. This was found to be true (188).

The stability on actinospectacin (69) correlated a copper chelate assay with biological activity, pointed out that the bioassay reliability was a function of buffer concentration, and kinetically observed a pKa *ca.* 12.

Other Studies.—Other kinetic studies on the stability of drugs that have been reported are the alkaline decomposition of glutethimide (195), the specific acid catalyzed decomposition of chlorothiazine (194) and diphenhydramine hydrochloride (146). The log *k*-pH profile has been given for procaine and tetracaine

(178) hydrolysis, substituted salicylic acid decarboxylation (191) as *p*-aminosalicylic acid (177, 179), and the solvolytic degradation of penicillin (21). The kinetics of formation of anhydrovitamin A for the alcohol and acetate of vitamin A have been investigated (99).

Thermal degradation of glucose solutions exhibited general acid-base catalysis with a rate dependence on substrate concentration (189). The heat of activation was determined from rate constants estimated by the initial slopes of the rate plots. The kinetics of glucose degradation in acid solution were also followed by the initial rate of formation of 5-hydroxymethylfurfural (90). The logarithm of the apparent rate constant varied linearly with the reciprocal of the dielectric constant, as predicted for a reaction between a positive ion and a dipole, as indicated by Eq. 26.

A catenary has been drawn for the pH dependency of the decomposition rate of oxophenarsine hydrochloride and Arrhenius parameters can be calculated from the data given (6).

The kinetics of bisulfite addition to compounds have been studied by Higuchi and Schroeter (100–102, 164, 166). They clearly show that not only can a pharmaceutical degrade in a solution and lose therapeutic potency but that a synthesis to a biologically inactive compound can also occur, as with epinephrine (166) and other benzyl derivatives (100). The probable reaction in these instances is to a benzyl sulfonic acid by reaction with a hydroxyl group (100–102). The bimolecular kinetics of epinephrine with sulfite ion were established, the heat of activation determined, and a quantitative expression derived for the given log *k*-pH profile (102). Bimolecular kinetics of salicyl alcohol reaction with bisulfite and/or sulfite ions were shown to be pH-dependent, with an appropriate predictive expression to explain the log *k*-pH profile (164). The Arrhenius parameters of the reaction were determined and ionic strength effects checked. This work has been recently considered by Schroeter in his discussion on sulfurous acid salts as pharmaceutical antioxidants (163).

The rates of hydrolysis of carbamate and carbonate esters (39, 40, 93) are of great importance in predicting the stability of carbamate esters of pharmaceutical utility (36). Nogami and coworkers have studied the hydrolysis of various quaternary nitrogen compounds (147, 148) and have observed specific acid-base and solvent catalysis, constructed the log *k*-pH profiles, and determined the Arrhenius parameters.

Prediction of Stability in Pharmaceutical Preparations.—A pharmaceutical preparation is frequently complex. Prior discussion in this paper on the prediction of stability has considered those circumstances where most of the variables are known and their specific effects on degradation can be quantified. This is not possible with the many formulations where prediction of market or shelf-life is needed. Typical examples of such a complex formulation are the multivitamin preparations which have been criticized for age on the market (26) and inadequate control, stability, and potency (27).

The legal, moral, economic, competitive, and public health reasons for the need of valid methods for the prediction and verification of drug stability in the marketed formulation have been outlined (26, 27, 44, 62, 63, 117, 136, 137).

Criticism has been leveled at rule-of-thumb methods of predicting shelf-life from accelerated testing (17, 44, 62, 63, 117, 126, 136) since, too frequently, such correlations have been intuitive, based on insufficient numbers of elevated temperatures or on empirical relations found in supposedly similar preparations (16, 63).

The Arrhenius relations of rate with absolute temperature of Eq. 38 and 39 show that any value proportional to the specific rate, k, would permit evaluation of the slope of the plot of log k *vs.* $1/T$ and, thus, prediction of stability at shelf or storage temperatures should be practical.

It is not necessary to determine the mechanisms of degradation. In many cases, the procedure is to follow some property of the degradation as a function of time and to linearize this function. The slopes of such linear plots may be used as estimates of the specific rates (k). The reproducibility of such a degradation rate function at the various elevated temperatures would validate the use of such functions in the prediction of degradation rates at lower temperatures (63).

The order of the degradation rate of a component in a complex preparation is defined as the power, n, of the concentration proportional to the rate of degradation, as is Eq. 1. In most studies on the thermal degradation of complex liquid preparations (46, 47, 63, 135) $n = 0$ or $n = 1$ has served satisfactorily to characterize the loss of initial assay with time within the errors of assay. Analogous procedures can be developed, however, for the other possible values of n. Autocatalytic degradation, however, may need special treatment and the thermal degradation of crystalline fumagillin in air gave apparent second-order kinetics (45).

The decrease in color of a multisulfa preparation was proportional to time (*i.e.*, zero order) at the various elevated temperatures studied (63). The logarithm of the slopes of such plots was linear with the reciprocal of the absolute temperatures of the studies and the values at the extrapolated room temperatures permitted prediction of the color stability at those temperatures. The thermal degradation of many vitamins in liquid multivitamin preparations has also been studied (46, 47, 135). Zero- and first-order plots (a typical example for thiamine hydrochloride is given in Fig. 1) were applicable. The Arrhenius plots, as in Fig. 15 for thiamine hydrochloride, permitted prediction of stability at the lower temperatures.

Although the equations of such plots can be estimated graphically (135), the employment of statistical procedures (22, 30, 31) determines the degree of confidence to be held in the predicted stability. These procedures have been outlined and applied by Garrett (46, 47), and were subsequently given stepwise (135). In general, the rate data are fitted by the method of least squares, as in Fig. 1, to give the best estimated rate constant and estimates of its error as well as estimates of error in the assay methods.

Similarly, the error in a predicted rate constant at a marketing temperature can be estimated (Fig. 15). The final verification of these predictive procedures has been given in detail in the literature (46, 135). An example is the solid line in Fig. 17 which predicted the thermal degradation of thiamine hydrochloride at 30° where the dashed lines encompass the standard error in the predicted thermal degradation and the 95% confidence limits of a single assay. The data were obtained from two separately prepared formulations maintained at 30° and assayed over a year. Another example is given in Fig. 18 and verifies the predicted thermal degradation of folic acid in a liquid multivitamin preparation at 25°. The upper curve was predicted for a modified preparation with an increased concentration of folic acid and was experimentally confirmed. This indicates that it may be used practicably in many cases to predict stability on the basis of one elevated temperature and the known Arrhenius slope.

This type of prediction may serve as an efficient control procedure as, when a batch of one component is introduced from a new source is increased in amount, or a manufacturing process is modified. This procedure is not applicable to two different components; Arrhenius slopes of such will differ even in the same preparation.

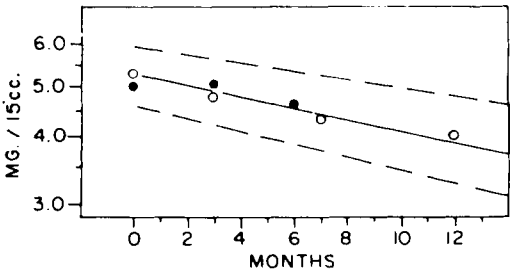


Fig. 17.—Verification of prediction of thiamine hydrochloride stability at room temperature. The solid line is the predicted thermal degradation rate of thiamine hydrochloride in a liquid multivitamin preparation A at 30°. The dashed lines encompass standard error in predicted values and the standard error of a single assay. O, Data from preparation A; ●, data from a similar preparation A′. [Figure 4 of Garrett, E. R., This Journal, **45**, 470(1956) (47).]

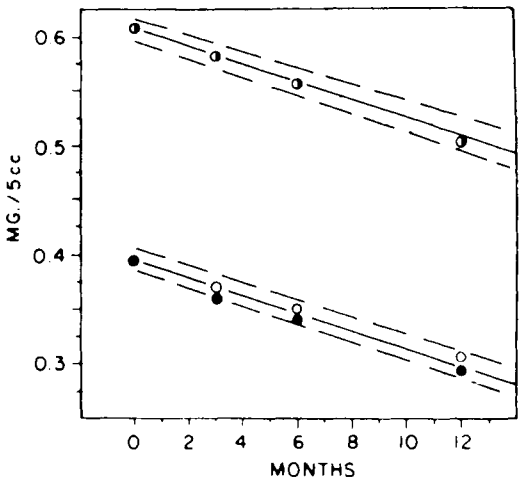


Fig. 18.—Verification of prediction of folic acid stability at room temperature. The solid lines are predicted thermal degradation rates of folic acid in liquid multivitamin preparation A at 25°. The dashed lines encompass standard error in predicted values and 95% confidence of a single assay. O, Data from preparation A; ●, data from a similar preparation B; ◑, data from a modified preparation C with increased initial concentration of folic acid and other vitamin components. [Figure 9 of Garrett, E. R., This Journal, **45**, 171(1956) (46)].

However, when the vehicle or formulation is entirely new, the rate of change of degradation rate of a component with temperature is not the same in both vehicles. Valid prediction is not possible from comparison at only one elevated temperature. The slopes of the Arrhenius equation must be determined anew.

The use of these physicochemical procedures for prediction of stability must be considered in the light of the controlling mechanisms of degradation (47). In the previous discussion components were considered that most probably degrade by solvolytic processes; *i.e.*, reactions

in solution and their heats of activation, as per Arrhenius slope, are generally in the range 10–30 Kcal./mole. Thus, advantage may be taken of significant increases in rate with temperature. However, if diffusion or photolysis are the rate-determining steps, the heat of activation is only of the magnitude 2–3 Kcal./mole and little advantage is gained by accelerated temperature studies in prediction, since the temperature effect on rate is small. In some cases, such rates may be accelerated by increases in pressure or light intensities.

Frequently the thermal degradation of polyhydroxylic materials and the subsequent effect on other components are of interest. However, it must be realized that the heats of activation for such pyrolysis are frequently of the magnitude of 50–70 Kcal./mole. Thus, rates of degradation which may be great at elevated temperatures may not be of any practical significance at the temperature of marketing and storage of the preparation.

Further discussions on design and practice of such predictive methods are given in the literature (44, 117) and the very models of ideal comprehensive stability testing laboratories for application of such techniques are also given (118, 119).

Other examples of the application of these predictive techniques in pharmaceutical formulations are for ascorbic acid in a liquid multivitamin emulsion containing fluoride (180), the kinetics of degradation of amyl nitrite ampuls (196, 197), and the stability of ergonovine maleate (139). Publications of interest in this regard are studies on the influence of the effect of heating and cooling time on prediction of shelf-life by kinetic principles and the Arrhenius equation at exaggerated temperatures (37). It has been shown, however, that for a series of samples subjected to elevated temperatures and when each sample is treated in the same manner (*i.e.*, heated and cooled), the calculated rates of degradation are identical to those measured under isothermal conditions (167).

CONCLUSIONS

The prediction of solvolytic stability of drugs in solution and in liquid pharmaceutical preparations is based on good science. The known quantitative relations between degradative rate and the extant variables (concentration, pH, solvent kind and dielectric constant, ionic strength, substrate pKa's, substrate reactive groups, temperature, solubility, nucleophiles, acids, bases, addend, etc.) have been shown to permit such prediction from a minimum of experiment and data and with a maximum of understanding and confidence. The procedures outlined provide additional pharmaceutical bonuses in the physicochemical characterization of substrates and products of kinetic transformations. They permit the correlation of biological activities and assays, assurance of valid assay methods, and estimations of their error, and the prediction of molecular, solvent, and addend changes to increase stability or increase physiological availability.

The future looms brightly for the prediction of stability of the complicated phenomena involved in photolytic and oxidative transformations. The complex stability problems of suspensions, emulsions, and ointments are leaving the backwoods of empirical correlations and rules-of-thumb and will shortly join the civilized communities of quantified prediction.

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Research Articles

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By T. L. FLANAGAN, L. W. REYNOLDS†, W. J. NOVICK, T. H. LIN, I. M. RONDISH, and E. J. VAN LOON

Chlorpromazine instilled intraduodenally in dogs was excreted in bile and urine as "free" and "bound" chlorpromazine and chlorpromazine sulfoxide. Based upon chromatographic studies and ultraviolet analysis, chlorpromazine and its sulfoxide undergo two types of binding. Strong alkaline treatment hydrolyzed one type while treatment with β-glucuronidase hydrolyzed both types. In urine, the concentration of the "bound" phenothiazines liberated by alkaline hydrolysis was 2–3 times as great as the "free" material. In bile, the concentration of this type of "bound" chlorpromazine and chlorpromazine sulfoxide was 10 to 15 times greater than the "free" forms of these materials.

VARIOUS investigators have reported on the urinary excretion of chlorpromazine and/or its metabolic products in man and experimental animals (1–9). Ross, Young, and Maass (10), Walkenstein and Seifter (11), and Fishman and Goldenberg (12) reported that the side chain which is attached to the phenothiazine nucleus is demethylated. Nadeau and Sobolewski (9) reported that β-glucuronidase treatment increases the amount of phenothiazine-like extractable material from human urine, and Lin, Reynolds, Rondish, and Van Loon (13) reported on the isolation and characterization of glucuronic acid conjugates of chlorpromazine in human urine. Fyodorov (14) administered chlorpromazine-S^{35} to dogs and found that the bile was distinguished by a high degree of radioactivity, especially 3–6 hours after administration.

The purposes of the present studies were (*a*) to obtain an insight into the distribution and

Received October 19, 1961, from the Research and Development Division, Smith Kline and French Laboratories, Philadelphia 1, Pa.
Accepted for publication December 14, 1961.
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metabolism of chlorpromazine by following its biliary and urinary excretion in the anesthetized dog and (*b*) by the use of chlorpromazine-S^{35}, to study further the metabolites present in urine and to elucidate the type of binding in urine.

EXPERIMENTAL

In the first experiments, mongrel female dogs weighing approximately 10 Kg. were anesthetized by intraperitoneal pentobarbital and operated so as to expose the duodenum and the common bile duct. A polyethylene cannula was inserted into the common bile duct about 1 in. above the major duodenal papilla and held in place by means of sutures. The open end of the cannula was brought to the outside of the animal and terminated in the collection tube. The cystic duct was clamped off to insure collection of only hepatic bile throughout the experiment. At the same time a catheter was inserted into the urinary bladder and all residual urine was drained and discarded.

One-half hour later, 200 mg. of chlorpromazine hydrochloride, dissolved in 10 ml. of saline, was administered to the animal intraduodenally. Bile samples were obtained at hourly intervals and urine samples at 2-hr. intervals for a period of 8 or 10 hours following drug administration. Each sample was analyzed individually for "free" and "bound" chlorpromazine and chlorpromazine sulfoxide by the method of Flanagan, *et al.* (15). The samples were not analyzed for glucuronic acid conjugates.

"Bound" chlorpromazine and "bound" chlorpromazine sulfoxide are the terms given to the chlorpromazine and chlorpromazine sulfoxide which can be extracted from the aqueous residue, following the extraction of the free components, after the aqueous residue is subjected to an alkaline hydrolytic treatment. The analytical procedure determined the ether-extractable chlorpromazine metabolites as either chlorpromazine or its sulfoxide. If demethylation of the side chain occurred, the demethylated components would be determined as chlorpromazine and/or chlorpromazine sulfoxide, since their absorption spectra are similar to the parent compounds.

In the next series of experiments the dogs received a daily oral dose of chlorpromazine hydrochloride for varying periods of time (Table I) prior to the operation. The operative dose was 200 mg. of chlorpromazine hydrochloride in 10 ml. of isotonic saline administered intraduodenally. Before the administration of the operative dose, control samples of bile and urine were obtained to determine any excretion due to the previous chlorpromazine priming.

In the experiments with chlorpromazine-S^{35}, each dog was anesthetized with pentobarbital and the bladder was emptied by inserting a catheter which was allowed to remain *in situ* for the subsequent collection of urine samples. Chlorpromazine hydrochloride, 25 mg./Kg., containing about 60 μc. of S^{35}, were injected into a surgically-exposed loop of the duodenum. Urine samples were collected every 2 hours.

Each sample was reduced *in vacuo* to about one-third of the original volume and 50–100 μl. spotted on $^3/_4$ inch strips of Whatman 3MM paper. The

TABLE I.—BILIARY AND URINARY EXCRETION IN PRIMED DOGS[a]

Dog	Collection Period, hr.	Daily Priming Dose of Chlorpromazine, mg.	No. of Days of Priming	% of Dose Recovered and Calculated as Chlorpromazine Hydrochloride — Free Chlorpromazine Bile	Free Chlorpromazine Urine	Free Chlorpromazine Sulfoxide Bile	Free Chlorpromazine Sulfoxide Urine	Total Recovery of Free Components, Bile and Urine	Bound Chlorpromazine Bile	Bound Chlorpromazine Urine	Bound Chlorpromazine Sulfoxide Bile	Bound Chlorpromazine Sulfoxide Urine	Total Recovery of Bound Components, Bile and Urine	Free *vs.* Bound Components
5	8	200	14	0.9	2.3	0.6	3.6	7.4	3.7	2.7	10.3	4.5	21.2	1:2.9
6	8	200	2	0.5	1.7	0.3	2.6	5.1	2.6	2.1	4.1	1.4	10.2	1:2.0
7	8	100	7	0.1	0.3	0.1	1.0	1.5	0.8	0.9	1.2	0.7	3.6	1:2.4
8	8	100	6	0.3	1.5	0.1	1.3	3.2	1.1	2.9	1.7	3.0	8.7	1:2.7

[a] Operative dose, 200 mg. of chlorpromazine hydrochloride intraduodenally.

TABLE II.—BILIARY AND URINARY EXCRETION IN DOGS FOLLOWING A SINGLE 200-MG. DOSE OF CHLORPROMAZINE HYDROCHLORIDE INTRADUODENALLY

Dog	Collection Period, hr.	% of Dose Recovered and Calculated as Chlorpromazine Hydrochloride — Free Chlorpromazine Bile	Free Chlorpromazine Urine	Free Chlorpromazine Sulfoxide Bile	Free Chlorpromazine Sulfoxide Urine	Total Recovery of Free Components, Bile and Urine	Bound Chlorpromazine Bile	Bound Chlorpromazine Urine	Bound Chlorpromazine Sulfoxide Bile	Bound Chlorpromazine Sulfoxide Urine	Total Recovery of the Bound Components, Bile and Urine	Free *vs.* Bound Components
1	8	0.3	1.1	<0.1	0.6	2.0	2.9	3.9	2.8	1.4	11.0	1:5.5
2	10	0.2	1.1	<0.1	1.4	2.7	2.4	2.3	1.9	1.8	8.4	1:3.1
3	10	0.3	1.1	0.1	1.6	3.1	3.6	3.7	5.4	3.2	15.9	1:5.1
4	10	0.1	0.9	<0.1	1.3	1.3	1.3	4.6	1.2	1.0	8.1	1:6.2

strips were developed in a descending direction with a solvent system consisting of 100 parts of isoamyl alcohol, 100 parts of water, 15 parts of ethanol, and 10 parts of formic acid.

A portion of the remaining urine was adjusted to pH 13 and extracted with ether to remove the "free" chlorpromazine and "free" chlorpromazine sulfoxide. The aqueous residue was divided into two fractions. One fraction was hydrolyzed with strong alkali, ether extracted, and the ether extract and the aqueous residue were chromatographed. The pH of the second fraction was readjusted to 5.0 and hydrolyzed with mammalian β-glucuronidase. After an ether extraction, the extract and the hydrolyzed urine were chromatographed in the same manner as the original urine.

The metabolites were detected by two independent procedures: (*a*) the chromatograms were sprayed with nitrite reagent as described by Flanagan, *et al.* (15), and (*b*) the developed strips were fed into a scanner (Nuclear-Chicago, model No. 1620-A) and the radioactive areas were detected in this manner.

In all the experiments the animals were maintained by a slow 5% dextrose-saline intravenous infusion and additional pentobarbital as required.

RESULTS AND DISCUSSION

In Table II are listed the per cent recoveries of chlorpromazine and chlorpromazine sulfoxide in bile and urine of four unprimed dogs which received an operative dose of 200 mg. of chlorpromazine hydrochloride intraduodenally. In bile, the concentrations of "bound" chlorpromazine and "bound" chlorpromazine sulfoxide were much greater than the concentrations of "free" chlorpromazine and "free" chlorpromazine sulfoxide. "Free" chlorpromazine sulfoxide was not obtained from the bile specimens of these dogs until 5–6 hours after administration of chlorpromazine; "bound" chlorpromazine sulfoxide was obtained in the first hour following administration of the drug. The ratio of "bound" chlorpromazine to "bound" chlorpromazine sulfoxide was approximately 1:1 in the test period for three of the dogs. The bile recovery of the bound components was 10–20 times greater than the recovery of the free components.

In urine the amount of "free" chlorpromazine and "free" chlorpromazine sulfoxide was much greater than that obtained in the bile. The recovery of "bound" chlorpromazine was from 2–5 times greater than the recovery of "free" chlorpromazine, while the "bound" chlorpromazine sulfoxide was 1.3 to 3 times greater than the "free" sulfoxide.

In the next experiment, four dogs received priming doses of chlorpromazine hydrochloride to see what effect priming would have upon the excretion pattern of the drug. In these animals the control bile samples contained measurable concentrations of "free" and "bound" chlorpromazine and chlorpromazine sulfoxide, although the last oral dose had been administered 18 hours prior to the operation. In the dog that was primed with 200 mg. of chlorpromazine hydrochloride for 14 days, there was an increased recovery of "free" and "bound" chlorpromazine and chlorpromazine sulfoxide. In these primed dogs the ratio of "bound" chlorpromazine to "bound" chlorpromazine sulfoxide in bile varied from 1:1.5 to 1:3, while in three of the unprimed dogs this ratio was 1:1.

The urinary values obtained for "bound" chlorpromazine on the primed animals were lower than the values obtained on the single-dose animals.

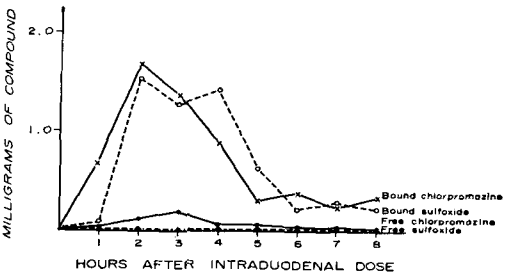


Fig. 1.—Dog No. 1, nonprimed biliary excretion.

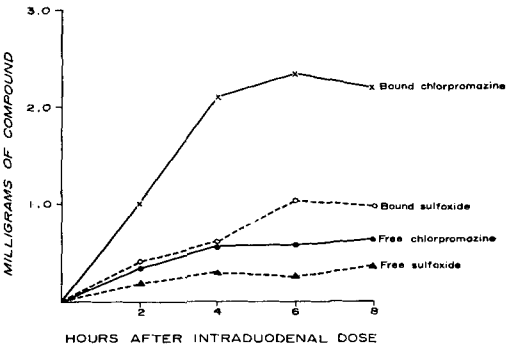


Fig. 2.—Dog No. 1, nonprimed urinary excretion.

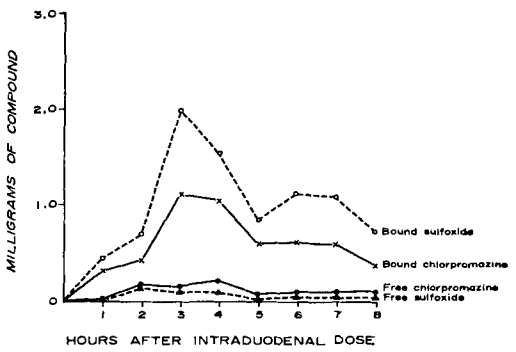


Fig. 3.—Dog No. 6, primed biliary excretion.

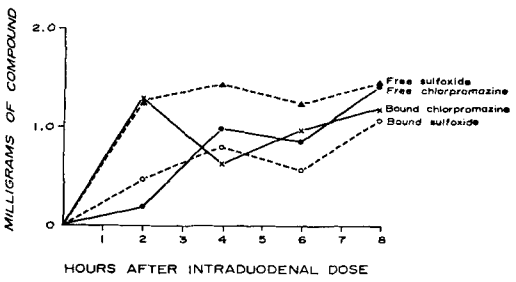


Fig. 4.—Dog No. 6, primed urinary excretion.

However, the ratio of "free" *vs.* "bound" components was increased in the primed animals due to the increased excretion of "free" components by these animals.

Figures 1 and 2 show the biliary and urinary time excretion curves of the four components in a nonprimed dog, while Figs. 3 and 4 illustrate the same curves for a primed dog. The patterns obtained on these two dogs are typical of those obtained on the other test animals.

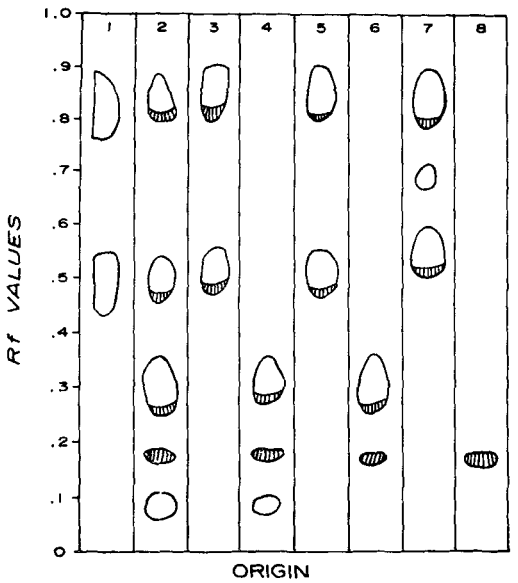


Fig. 5.—Col. 1, Chlorpromazine and chlorpromazine sulfoxide standards; Col. 2, whole urine; Col. 3, ether extract of whole urine (alkaline side); Col. 4, aqueous residue; Col. 5, fraction 1, ether extract after alkaline hydrolysis; Col. 6, aqueous residue; Col. 7, fraction 2, ether extract after β-glucuronidase hydrolysis; Col. 8, aqueous residue.

In the chromatographic studies with chlorpromazine-S^{35} on noncannulated, primed and unprimed dogs, four radioactive peaks were observed. The two peaks following the solvent front corresponded to chlorpromazine and chlorpromazine sulfoxide. The two remaining peaks were attributed to bound metabolites of these compounds. No attempt was made to separate chlorpromazine and chlorpromazine sulfoxide from their demethylated components as did Fishman and Goldenberg (12). Typical chromatographic patterns obtained on urine from dogs administered chlorpromazine-S^{35} by spraying the strips with nitrite reagent are shown in Fig. 5.

In all the experiments, the R_f values obtained for the metabolites by the use of the color developing spray corresponded to the R_f values obtained radiochemically.

The chromatographic patterns of chlorpromazine and chlorpromazine sulfoxide are presented in Col. 1 (Fig. 5). Column 2 shows the chromatographic pattern obtained for whole urine; the spots between the two "bound" metabolic spots represent an endogenous urinary constituent which was obtained with this spray for blank urine specimens. The dark areas behind the chlorpromazine and chlorpromazine sulfoxide were always present and they were purple in color as compared to a light pink shade of the pure compounds. The darker areas did not show increased radioactivity over that obtained for the pink shaded areas. Column 3 shows the pattern for the ether extract of whole urine made alkaline. The "bound" material remained in the aqueous phase (Col. 4).

In regard to Col. 4, it was believed initially that the two "bound" metabolites corresponded to "bound" chlorpromazine and "bound" chlorpromazine sulfoxide. However, following alkaline hydrolysis and subsequent ether extraction, the spot having an R_f of approximately 0.1 disappeared from the aqueous residue, while the R_f 0.3 spot remained (Col. 6). The chromatographic pattern obtained for the ether extract (Col. 5), as well as ultraviolet analysis of this extract, showed both chlorpromazine and chlorpromazine sulfoxide to be present. The R_f 0.1 spot was a mixture of "bound" chlorpromazine and "bound" chlorpromazine sulfoxide, and the type of binding is such that it can be broken by strong alkaline hydrolytic treatment. The "bound" components in this area did not resolve themselves into separate and distinct entities. The separation was not as sharp as that obtained by Lin, *et al.* (13), with human urine.

The chromatogram obtained for the aqueous residue after hydrolysis with β-glucuronidase and subsequent extraction with ether is presented in Col. 8. Only the endogenous spot remained; β-glucuronidase hydrolyzed both "bound" areas. In the ether extract (Col. 7) three spots appeared; two of the spots corresponded to chlorpromazine and chlorpromazine sulfoxide, the small spot in between may be one of the metabolites resulting from partial or total demethylation of the side chain.

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Use of Isolated Strips of Cat Spleen for the Assay of α-Adrenergic Blocking Compounds

By ROBERT K. BICKERTON, WILLIAM T. ROCKHOLD, and EDWIN R. MICALIZZI

The response of isolated strips of cat spleen to epinephrine, norepinephrine, and acetylcholine was studied. It was found that spleen strips suspended in glucose-deficient Tyrode's solution exhibited marked sensitivity to the catecholamines and were relatively insensitive to acetylcholine. This differential sensitivity was utilized to advantage in the development of an *in vitro* assay procedure for α-adrenergic blocking agents. In addition, the method offers a qualitative index to the degree of tissue receptor binding of the antagonists.

MANY *in vitro* preparations have been used for the assay of sympathetically active compounds. Brugger (1), Rothlin and Brugger (2), and Rothlin (3) used the isolated seminal vesicle of the guinea pig to assay for the adrenolytic effects of the ergot alkaloids. Stone and Loew (4) used this same preparation as an assay for antiadrenaline drugs and Leitch, Liebig, and Haley (5) used the rat's seminal vesicle for the assay of certain sympatholytic drugs. Burn, Finney, and Goodwin (6) described several *in vitro* techniques for the assay of adrenaline and noradrenaline. Many past reports, and more recently those of Scott (7), Burn and Rand (8, 9), Daly and Scott (10), and Brandon and Rand (11) have described studies concerning the response of the mammalian spleen, both *in situ* and *in vitro*, to a number of drugs. None of these reports has described the use of this organ for the assay of adrenergic blocking compounds. This report deals with the response of isolated strips of cat spleen to epinephrine, norepinephrine, and acetylcholine, and the use of this organ for the assay of adrenergic blocking agents.

EXPERIMENTAL

Methods.—In all experiments, cats weighing 2 to 3 Kg. and unselected as to age or sex were used. The animals were anesthetized by an intraperitoneal injection of 0.7 ml./Kg. of diallylbarbituric acid-urethane.[1] The spleen was exteriorized through a lateral abdominal incision and then removed by section of all vasculature at the junction of splenic pedicle and dorsal hilus. The spleen was then rinsed and placed in warmed Tyrode's solution containing one-half the usual amounts of sodium bicarbonate and anhydrous dextrose. The sides and ends of the spleen were trimmed to leave a central rectangular strip measuring approximately 9 × 1.5 cm. This strip was divided into two equal portions, each measuring 4.5 × 1.5 cm., and each half was placed into a separate 70-ml. muscle bath containing oxygenated (95% oxygen, 5% carbon dioxide) Tyrode's solution of the previously mentioned composition. The bath was maintained at 39°. Each spleen strip was connected to an isotonic ink-writing lever so that contractions could be recorded on a kymograph. A magnification ratio of 13:1 was used and the strips were placed under constant tension of 5.0 Gm. Acetylcholine chloride, *l*-epinephrine bitartrate, and *l*-norepinephrine bitartrate were dissolved in 0.9% sodium chloride just before use. Ergotamine tartrate and tolazoline hydrochloride were prepared by dilution of the commercially available ampuls with 0.9% sodium chloride prior to use. Dibenamine hydrochloride was prepared as a 5% stock solution in propylene glycol and diluted with 0.9% sodium chloride just prior to use. Guanethidine sulfate was dissolved in dilute hydrochloric acid solution and buffered to pH 6 with sodium bicarbonate. Final dilutions of guanethidine were made with 0.9% sodium chloride solution. Doses of all drugs were added to the baths in 0.35- to 2.1-ml. volumes except in the assay procedure where all doses of drugs were added in 1.0-ml. volumes.

In performance of the assay, *l*-epinephrine was added to each bath to give a concentration of 0.5 mcg./ml. and kept in contact with the spleen strip for 5 minutes. During this time maximal contraction to this dose occurred. The epinephrine was then drained and the strip washed and allowed to equilibrate for 10 minutes, and then the wash was repeated. If complete relaxation of the strips did not occur within this interval, the writing lever was manually returned to base line. Five minutes after the last wash, *l*-epinephrine was again added and the above procedure repeated. Epinephrine was added at 20-minute intervals until four consecutive responses were obtained which did not vary more than 5% from their mean. Immediately after the second wash from the last control response, the antagonists were added to the baths in varying concentrations and allowed to remain in contact with the strip for 5 minutes. Without further washing, *l*-epinephrine was again added to the bath and the contraction recorded. The strip was washed as described and a minimum of two more responses to epinephrine were obtained at the usual 20-minute intervals in order to determine how readily the antagonists were removed from their receptor sites. Only one dose of an antagonist was administered to any one spleen strip, with the exception of tolazoline which was administered in repeated doses to

Received December 18, 1961, from the Section of Pharmacology, Eaton Laboratories, Norwich, N. Y.
Accepted for publication January 3, 1962.
[1] Dial-Urethane (Ciba)

three strips. Per cent inhibition of epinephrine response from a given dose of antagonist was calculated from the difference in contraction heights of the mean of the four consecutive responses prior to the antagonist, and the response to epinephrine immediately following the antagonist. ED_{50} values for the antagonists were determined with the aid of an IBM 1620 digital computer programmed to determine the log-dose, probit-inhibition least squares regression line, its slope, and 95% confidence limits for the slope for each antagonist. All values were obtained at printout.[2]

The effect of time of contact of the antagonist with the spleen strip was studied in two strips from the same animal. Tolazoline and dibenamine were selected as the antagonists. The standard assay procedure was carried out excepting that the antagonists were allowed to remain in contact with the spleen for only 5 minutes in one strip and for 10 minutes in the remaining strip.

RESULTS

Effect of Epinephrine, Norepinephrine, and Acetylcholine.—Addition of *l*-epinephrine or *l*-norepinephrine to the spleen strips in varying concentrations produced contractions which were initially rapid, and then followed by a slow component which reached a maximum within 5 minutes. The response of the spleen strips to a given dose of these catecholamines increased in magnitude over the first four or five responses and then remained relatively uniform over the next several hours. Dose-response curves obtained from uniformly reacting strips were found to be logarithmically linear throughout a concentration range of 0.05 to 1.0 mcg. free base/ml. Some variation in reactivity to these catecholamines was found with different strips, although all spleen strips tested were more sensitive to epinephrine than to norepinephrine (see Fig. 1).

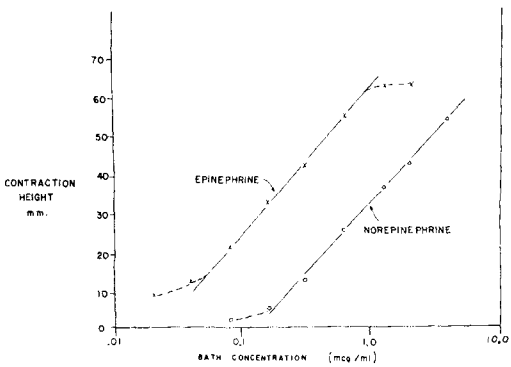


Fig. 1.—Log dose response curves for epinephrine and norepinephrine obtained from uniformly reacting cat spleen strip.

The contractile response of isolated cat spleen strips to acetylcholine, when present, was found to be moderate throughout a concentration range of 10 to 430 mcg./ml. When administered at 5-minute intervals, tachyphylaxis to acetylcholine rapidly developed, often after a single dose, and almost always within four repeated doses, although longer intervals between doses delayed the onset of tachyphylaxis (see Fig. 2).

Assay of Adrenergic Blocking Agents.—Table I lists the ED_{50} values for the α-adrenergic blocking agents dibenamine, ergotamine, and tolazoline. Since the slopes of the log-dose inhibition curves were not parallel,[3] potency comparisons were based

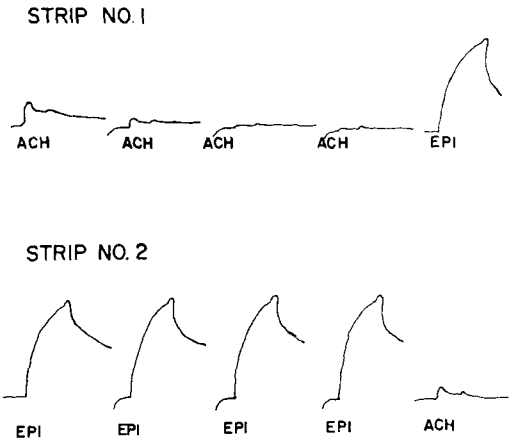


Fig. 2.—Development of tachyphylaxis to acetylcholine while the responses of spleen strip to epinephrine are unimpaired. EPI, 35 mcg. *l*-epinephrine; ACH, 700 mcg. acetylcholine chloride.

upon the ED_{50} values. With respect to the ED_{50} values, the following relationship, in order of decreasing potency, was found: ergotamine > dibenamine > tolazoline. Guanethidine, a sympatholytic agent, in bath concentrations ranging from 10 to 200 mcg./ml., did not inhibit, but in some trials enhanced epinephrine-induced contractions of isolated strips of cat spleen. Guanethidine alone exerted intrinsic contractile activity on the spleen strips in concentrations of 50 to 200 mcg./ml.

Degree of Tissue Receptor Binding of the Antagonists.—Since this assay procedure used repetitive washing and stimulation of the spleen strip by epinephrine, the method offers an index to the degree of binding of the antagonists at tissue receptors. Figure 3 shows the effects of the antagonists when added to the bath at their approximate ED_{50} concentration and the effects of repeated washing and stimulation by epinephrine. It can be seen that dibenamine produced progressive inhibiton of epinephrine-induced contraction seven though the solution containing the antagonist had been drained from the bath. Maximum inhibition of epinephrine by a given dose of ergotamine occurred within 5 minutes and was relatively prolonged. Tolazoline produced an immediate (within 5 minutes) inhibition of epinephrine but was more readily removed from binding sites by fewer washing cycles. The degree of receptor binding of the antagonists occurred in the following order of decreasing affinity for tissue receptors: dibenamine > ergotamine > tolazoline.

In determining the effects of antagonist contact time on the inhibition of epinephrine, it was found

[2] The authors wish to express their sincere appreciation to Dr. R. L. Beinert and G. V. O'Bleness for the statistical analysis used in this study.

[3] Slope parallelism was tested by the method of Davies (12).

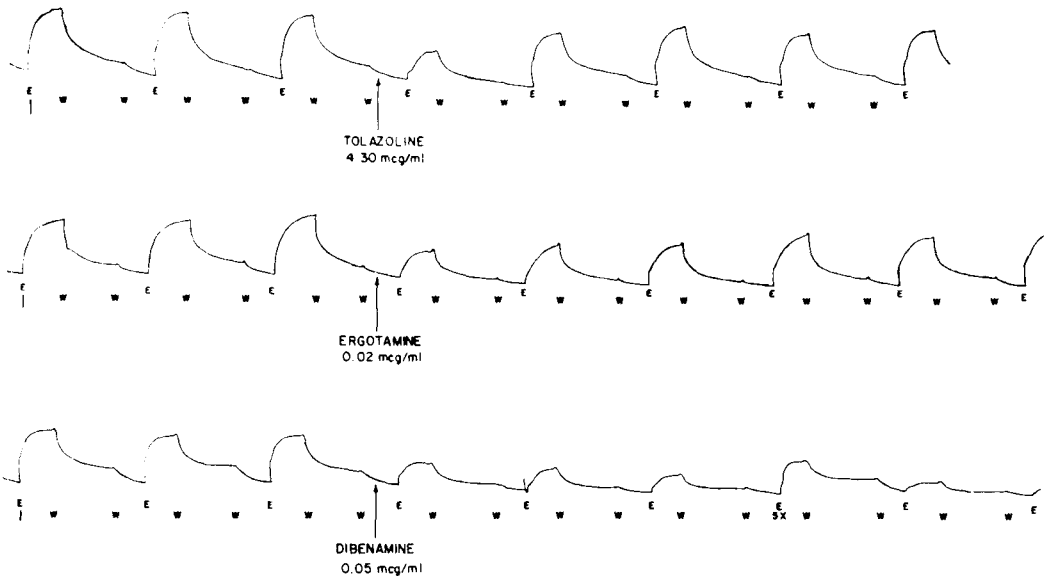


Fig. 3.—The effects of the antagonists when administered at their approximate ED_{50} concentrations on contractile responses of is olatedcat spleen to epinephrine (see text). E, Addition of 35 mcg. of epinephrine; W, wash.

that an increase in the time during which tolazoline was in contact with the spleen did not increase the inhibition of epinephrine by this antagonist but rather a decrease was observed. However, dibenamine produced greater inhibition after 10 minutes contact time than after 5 minutes contact (see Table II).

General Observations.—Throughout this study it has been our experience that the use of isolated strips of cat spleen for the assay of adrenergic blocking agents offers a major advantage over previous *in vitro* techniques in the relative durability of this organ. No special care in handling or removal from the animal is required. The organ is easily accessible through a small lateral incision which may be sutured after removal of the spleen. We have refrigerated excised spleen in Tyrode's solution containing a small amount of blood and have found that refrigeration for as long as 48 hours only slightly diminished the sensitivity to epinephrine. Longer periods of time resulted in greater decrease in sensitivity.

DISCUSSION

The contractile response of isolated cat spleen strips to *l*-epinephrine and *l*-norepinephrine was found to be logarithmically linear over a concentration range of 0.05 to 1.0 and 0.2 to 5.0 mcg. free base/ml., respectively. Responses to these catecholamines were reproducible over a period of many hours while those to administered acetylcholine under the conditions imposed by the assay were not. The order of potency of the three test antagonists was not in complete agreement with the data presented by Nickerson (13) who states that in potency, the ergot alkaloids occupy, in most instances, an intermediate position between the β-haloalkylamines and the imidazoline derivatives. The higher relative potency of ergotamine in our preparation could be explained on the basis of temporal relationships since it is known that the onset of dibenamine blockade is not immediate and that the degree of adrenergic blockade progresses with time up to the point of maximum effect. In our preparation, the dibenamine was in contact with the spleen strips for only 5 minutes. It is conceivable, therefore, that maximum blockade did not occur during this time. Further support of this suggestion was obtained from the fact that progressive inhibition did occur with dibenamine-treated strips, even though the bath containing the dibenamine had been drained and replaced with fresh Tyrode's solution. On the other hand, blockade produced by ergotamine was immediate and the degree of blockade was, therefore, accurately determined after 5 minutes contact time. The high potency of dibenamine and ergotamine in comparison to tolazoline is in agreement with Nickerson (13).

The finding that guanethidine did not inhibit epinephrine is in agreement with the data of Maxwell, *et al.* (14), who demonstrated the purely sympatholytic (as opposed to adrenolytic) action of this agent.

In considering the degree of tissue receptor binding of the antagonists, our results are in agreement with the data described by others (13). The progressive inhibition produced by dibenamine is similar to that which occurs in the intact animal and has been explained on the basis of the transformation from the equilibrium to the nonequilibrium type of blockade, presumably through the formation of ethylene immonium and/or vinyl intermediates, which then react chemically with cell constituents to produce a prolonged blockade. In this same regard, Axelrod *et al.* (15), reported that dibenamine did not disappear rapidly from the body of dogs but was extensively localized in body fat. These authors also found a close correlation between the degree of adrenergic blockade and the concentration of dibenamine in fatty tissue.

The rapidity with which tolazoline can be removed

TABLE I.—RESULTS OF THE ASSAY

Drug	No. Strips	Dose Range, mcg./ml.	ED_{50}, mcg./ml. (95% Confidence Limits)	Slope	95% Confidence Limits
Dibenamine	14	0.01 –1.0	0.05 (0.04–0.06)	2.4	±0.8
Tolazoline	11	1.0 –24.0	4.30 (3.37–5.50)	1.8	±0.5
Ergotamine	18	0.001–0.8	0.02 (0.01–0.03)	0.8	±0.2
Guanethidine	2	1.0 –200.0	>200	0.0	...

TABLE II.—EFFECT OF CONTACT TIME ON EPINEPHRINE INHIBITION

	Contact time	
	5 min.	10 min.
Tolazoline	52.8[a]	46.9[a]
Dibenamine	37.6	47.4

[a] Per cent inhibition.

from tissue receptors suggests that the binding forces involved are small and that removal from binding sites is dependent upon the distribution coefficient of the substance between tissue receptor and bathing medium. Brodie, *et al.* (16), have shown that tolazoline is only 23% plasma protein bound in the intact dog. These authors also found a relatively low degree of tissue localization of this agent and a relatively short biological half-life.

We have found this preparation to be an excellent tool for the investigation of drug action at adrenergic receptors. The relative ease in handling this tissue offers a distinct advantage over other methods. Investigations of the mechanism of action of a number of drugs on this organ are in progress.

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Synthesis of Some N- and S-Substituted Derivatives of 2-Aminobenzenethiol

By CHARLES S. DAVIS, GLENN L. JENKINS, ADELBERT M. KNEVEL, and CHARLES PAGET

N- and S-substituted derivatives of 2-aminobenzenethiol were prepared as intermediates in the synthesis of analogs of the phenothiazine tranquilizers. A new synthesis for the preparation of the benzothiazine ring is reported. The ring cleavage of 2,2-pentamethylenebenzothiazoline with alkylating agents is discussed.

IN RECENT years derivatives of 2-aminobenzenethiol have attracted attention in the development of medicinal agents. Gialdi and Baruffini (1) synthesized a series of 2-aminophenyl alkyl sulfides as potential fungicides. Burger, *et al.* (2), prepared N-substituted 2-aminophenyl aryl sulfides and found some of them to be radiation protective agents. We have undertaken the synthesis of analogs of the phenothiazine tranquilizers and found it necessary to prepare N- and S-substituted 2-aminobenzenethiols as intermediates. Bialdi and Baruffini (3) had prepared similar thiols by reducing 2,2′-dinitrodiphenyldisulfide with glucose to 2-nitrobenzenethiol. 2-Nitrobenzenethiol was then substituted with an alkyl bromide to give 2-nitrophenyl alkyl sulfide. This paper reports a one-step procedure of reacting commercial 2-aminobenzenethiol with alkyl halide in the presence of potassium hydroxide solution. Not only are the compounds prepared in fewer steps than those of Gialdi and Baruffini, but the yields of the corresponding products are

Received January 6, 1962, from the Research Laboratories, School of Pharmacy, Purdue University, Lafayette, Ind.
Accepted for publication January 25, 1962.

TABLE I.—RESULTS OF THE ASSAY

Drug	No. Strips	Dose Range, mcg./ml.	ED_{50}, mcg./ml. (95% Confidence Limits)	Slope	95% Confidence Limits
Dibenamine	14	0.01 –1.0	0.05 (0.04–0.06)	2.4	±0.8
Tolazoline	11	1.0 –24.0	4.30 (3.37–5.50)	1.8	±0.5
Ergotamine	18	0.001–0.8	0.02 (0.01–0.03)	0.8	±0.2
Guanethidine	2	1.0 –200.0	>200	0.0	...

TABLE II.—EFFECT OF CONTACT TIME ON EPINEPHRINE INHIBITION

	Contact time	
	5 min.	10 min.
Tolazoline	52.8[a]	46.9[a]
Dibenamine	37.6	47.4

[a] Per cent inhibition.

from tissue receptors suggests that the binding forces involved are small and that removal from binding sites is dependent upon the distribution coefficient of the substance between tissue receptor and bathing medium. Brodie, *et al.* (16), have shown that tolazoline is only 23% plasma protein bound in the intact dog. These authors also found a relatively low degree of tissue localization of this agent and a relatively short biological half-life.

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Synthesis of Some N- and S-Substituted Derivatives of 2-Aminobenzenethiol

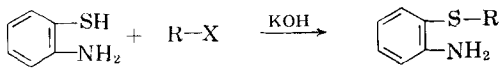
By CHARLES S. DAVIS, GLENN L. JENKINS, ADELBERT M. KNEVEL, and CHARLES PAGET

N- and S-substituted derivatives of 2-aminobenzenethiol were prepared as intermediates in the synthesis of analogs of the phenothiazine tranquilizers. A new synthesis for the preparation of the benzothiazine ring is reported. The ring cleavage of 2,2-pentamethylenebenzothiazoline with alkylating agents is discussed.

IN RECENT years derivatives of 2-aminobenzenethiol have attracted attention in the development of medicinal agents. Gialdi and Baruffini (1) synthesized a series of 2-aminophenyl alkyl sulfides as potential fungicides. Burger, *et al.* (2), prepared N-substituted 2-aminophenyl aryl sulfides and found some of them to be radiation protective agents. We have undertaken the synthesis of analogs of the phenothiazine tranquilizers and found it necessary to prepare N- and S-substituted 2-aminobenzenethiols as intermediates. Bialdi and Baruffini (3) had prepared similar thiols by reducing 2,2′-dinitrodiphenyldisulfide with glucose to 2-nitrobenzenethiol. 2-Nitrobenzenethiol was then substituted with an alkyl bromide to give 2-nitrophenyl alkyl sulfide. This paper reports a one-step procedure of reacting commercial 2-aminobenzenethiol with alkyl halide in the presence of potassium hydroxide solution. Not only are the compounds prepared in fewer steps than those of Gialdi and Baruffini, but the yields of the corresponding products are

Received January 6, 1962, from the Research Laboratories, School of Pharmacy, Purdue University, Lafayette, Ind.
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TABLE I.—2-AMINOPHENYL ALKYL SULFIDES



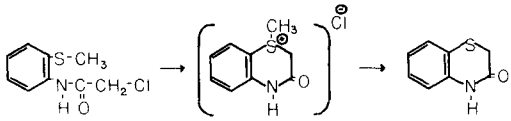
R	Yield, %	B.P., °C. (mm.)	M.P., °C.	Lit. M.p., °C. (3)
$-CH_2CH=CH_2$	75	84–86 (0.40)	142–143[g]	143–144
$-CH_2C_6H_5$	90	190–192 (0.15)	43–44	45
$-C_5H_{11}$-n	77	98–99 (0.45)	151–153[g]	152–153
$-CH_3$	95	121–123 (18)	189–190[g]	188–189
$-(CH_2)_3-NO$	67	165–166 (0.35)	54–55	[a]
$-(CH_2)_2-O-CH=CH_2$	50	109–110 (.35)	...	[b]
$-(CH_2)_2-N-(C_2H_5)_2$	78	130–132 (0.7)	...	[c]
$-(CH_2(CH_3)-CH-N-(CH_3)_2$	80	106–108 (0.5)	...	[d]
$-(CH_2)_2-N-(i-C_3N_7)_2$	75	120–122 (0.5)	...	[e]
$-CH_2)_3-N-(C_2H_5)_2$	76	162–164 (2.0)	198–200[g]	[f]

[a] *Anal.*—Calcd. for $C_{13}H_{20}N_2OS$: S, 12.71. Found: S, 12.57. [b] *Anal.*—Calcd. for $C_{10}H_{13}NOS$: S, 16.41. Found: S, 16.63. [c] *Anal.*—Calcd. for $C_{12}H_{20}N_2S$: S, 14.30. Found: S, 14.26. [d] *Anal.*—Calcd. for $C_{11}H_{18}N_2S$: S, 15.23. Found: S, 15.18. [e] *Anal.*—Calcd. for $C_{14}H_{24}N_2S$: S, 12.66. Found: S, 12.73. [f] *Anal.*—Calcd. for $C_{13}H_{22}N_2S$: S, 13.45. Found: S, 13.95. [g] Melting point of the hydrochloride salt.

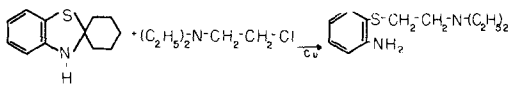
significantly higher. The compounds thus obtained are listed in Table I.

2-(2′-Diethylaminoethylamino)phenyl methyl sulfide (IV) was prepared by two methods. 2-Aminophenyl methyl sulfide (I) was alkylated directly with 2-diethylaminoethyl chloride hydrochloride in the presence of a copper catalyst. The compound IV was also synthesized by a method suggested by Burger (2). 2-Aminophenyl methyl sulfide (I) was reacted with chloroacetyl chloride to give 2-(2′-chloroacetamido)phenyl methyl sulfide (II) which was combined with diethylamine to give 2-(2′-diethylaminoacetamido)-phenyl methyl sulfide (III). This was reduced to 2-(2′-diethylaminoethylamino)phenyl methyl sulfide (IV) with lithium aluminum hydride.

2 - (2′ - Diethylaminoethylamino)benzenethiol (V) was synthesized by two procedures. The first was the alkaline hydrolysis of 2-oxo-3-(2′-diethylaminoethyl)benzothiazoline which gave a 50% yield of V. Alternately, the reduction of 2-(2′-diethylaminoethylamino)phenyl methyl sulfide (IV) with lithium in methylamine (4) gave 28% yield of V. In an attempt to purify 2-(2′-chloroacetamido)phenyl methyl sulfides (II) by distillation, methyl chloride was split out to produce 3-oxo-1,4-benzothiazine in 65–75% yields.



The spiro structure, 2,2-pentamethylenebenzothiazoline (VI) was prepared by a modification of the procedure outlined by Elderfield and McClenachen (5). The alkylation of VI was attempted with 3-diethylaminoethyl chloride and a copper catalyst. The major product was the corresponding sulfide and none of the N-alkylated material. Since this ring cleavage was not expected, VI was subjected to the copper catalyst alone. Starting material was recovered and none of the cleavage product could be found. Elderfield (5) reported the cleavage of the pentamethylene ring at high temperatures but not the heterocyclic ring.



EXPERIMENTAL[1]

2-Aminophenyl Methyl Sulfide (I).—To a solution of 34 Gm. (0.6 mole) of potassium hydroxide in sufficient water to just bring about solution, was added 37.5 Gm. (0.3 mole) of 2-aminobenzenethiol.[2] After cooling the mixture to room temperature, 43 Gm. (0.3 mole) of methyl iodide[3] was added slowly with rapid stirring. The rate of addition was such that the boiling temperature of methyl iodide was not exceeded. Water, 1000 ml., was added and an oil separated which was washed with 50 ml. of water and dried. The oil was distilled at 121–123° (18 mm.) to yield 95% of product.

The physical constants of the various 2-aminophenyl alkyl sulfides prepared by the above method are described in Table I.

2 - (2′ - Diethylaminoethylamino)phenyl Methyl Sulfide (IV).—*Method A.*—A suspension of 9.5 Gm. (0.25 mole) of lithium aluminum hydride in 30 ml. of dry ether was stirred and refluxed for 1 hour. To the above suspension was added, dropwise, a solution of 21.0 Gm. (0.1 mole) of 2-(2′diethylaminoacetamido)phenyl methyl sulfide (III) in dry ether over 25 minutes. The mixture was refluxed and stirred for 15 hours. The unreacted lithium aluminum hydride was then decomposed,

[1] All melting points are uncorrected. Analyses were performed by the Alfred Bernhardt, Mikroanalytisches Laboratorium in Max-Planck-Institut fur Kohlenforschung, Mulheim (Ruhr), Germany.

[2] 2-Aminobenzenethiol was supplied by American Cyanamid Co.

[3] It was found that the chloro or bromo compounds reacted as readily as the iodo compound.

with cooling, by dropwise addition of 2 ml. of ethyl acetate followed by 3 ml. of water. After vigorous stirring for 2 hours, the mixture was filtered by vacuum. The filtrate was treated with ethereal hydrogen chloride until the solution was neutral to litmus. The ethereal solution was made basic with 5% potassium hydroxide solution. The aqueous layer was extracted with ether and dried. The resulting oil was distilled at 106–107° (0.35 mm.) to yield 7.9 Gm. (40%).

Anal.—Calcd. for $C_{18}H_{22}N_2S$: S, 13.45. Found: S, 13.56.

Method B from 2-Aminophenyl Methyl Sulfide (I).—Twenty-one grams (0.15 mole) of I and 32 Gm. of anhydrous potassium carbonate were mixed with 20 ml. of dry benzene. To the slurry were added 18.6 Gm. (0.1 mole) of 2-diethylaminoethyl chloride hydrochloride and 0.5 Gm. of copper bronze[4] in 70 ml. of dry benzene. This mixture was stirred and refluxed for 10 hours. The brown mixture was filtered and the residue was washed with benzene. The product was distilled at 123–124° (1.5 mm.) to yield 4.2 Gm. (15%) of material identical to that prepared by method A.

2 - (2′ - Chloroacetamido)phenyl Methyl Sulfide (II). A solution consisting of 13.9 Gm. (0.1 mole) of 2-aminophenyl methyl sulfide (I) and 9.9 Gm. (0.125 mole) of dry pyridine in 200 ml. of dry ether was cooled to 0°. To this mixture was added an ethereal solution of 11.9 Gm. (0.105 mole) of chloroacetyl choride. After standing for 2 hours, the ethereal solution was washed twice with 100 ml. of 5% hydrochloric acid and then dried. The ether was removed and the orange oil crystallized upon standing. After recrystallization from aqueous ethanol, the waxy crystals melted at 43–44° to give 12.5 Gm. of product.

Anal.—Calcd. for $C_9H_{10}ClNOS$: S, 14.87. Found: S, 14.62.

When distillation of 5 Gm. (0.23 mole) of 2-(2′-chloroacetamido)phenyl methyl sulfide (II) was attempted, 3.5 Gm. (75%) of 3-oxo-1,4-benzothiazine resulted. The conversion was brought about by heating II in oil bath at 195° under 20 mm. pressure for 30 minutes. Upon cooling, the mixture solidified and 50 ml. of methanol was added. The alcoholic solution was boiled with Norite and filtered. Upon cooling the filtrate, pale yellow crystals were formed that melted at 177–178° [lit. (6) m.p. 179°.] The infrared spectrum was identical to an authentic sample.

2 - (2′ - Diethylaminoacetamido)phenyl Methyl Sulfide (III).—To a solution of 46 Gm. of 2-(2′-chloroacetamido)phenyl methyl sulfide (II) dissolved in 200 ml. of ether was added 31.2 Gm. of diethylamine in 40 ml. of ether. The ethereal solution was refluxed for 12 hours and then washed with 500 ml. of water. The ethereal solution was dried and distilled. The fraction distilling at 151–153° (3 mm.) was taken as product. The yield was 54 Gm. (85%); m.p. hydrochloride salt, 149–152°.

[4] No. 44 Bronze powder manufactured by Sunbrite Mfg. Co., St. Louis, Mo.

Anal.—Calcd. for $C_{13}H_{20}N_2OS$: S, 12.71. Found: S, 12.85.

2 - (2′ - Diethylaminoethylamino)benzenethiol (V).—A methylamine solution of 0.58 Gm. of lithium wire was added slowly to 10 Gm. of 2-(2′-diethylaminoethylamino)phenyl methyl sulfide (IV) in 150 Gm. of methylamine. To accomplish this, the methylamine was refluxed through a thimble containing the lithium wire cut into small pieces. The thimble was suspended above the reaction flask and under a dry ice condenser so that the methylamine vapors could travel from the reaction flask into the dry ice condenser. The thimble consisted of a 6-in. test tube tapered at the bottom and fitted with a length of glass tubing sufficiently long to extend well down into the neck of the reaction flask. [See (4) for a detailed description of apparatus used.] A small loose plug of glass wool placed in the bottom of the thimble kept small pieces of undissolved metal from dropping into the flask. After all of the lithium had dissolved, the methylamine was allowed to evaporate overnight and the residue was dissolved in water. The aqueous solution was neutralized with 10% acetic acid and extracted with ether. The ethereal solution was dried and concentrated to give an orange solid which melted at 103–105° [lit. (7) m.p. 100–102°]. Yield, 2.7 Gm. (28%).

Attempted Alkylation of 2,2-Pentamethylenebenzothiazoline.—In 200 ml. of dry toluene, 20.5 Gm. (0.1 mole) of 2,2-pentamethylenebenzothiazoline, 18.6 Gm. (0.1 mole) of diethylaminopropyl chloride hydrochloride, 40 Gm. of anhydrous potassium carbonate, and 0.1 Gm. of copper powder were refluxed with stirring for 24 hours. The reaction was allowed to cool and the solid residue filtered. The toluene was removed from the filtrate and the residue was distilled at 124–135° (0.1 mm.).

The distilled liquid mixed with petroleum ether (30:60) was allowed to pass through a column of alumina. The eluate was concentrated and the residue distilled at 162° (2 mm.). This material was found to be (3-diethylaminopropyl)-2-aminophenyl sulfide.

The product has an infrared spectrum identical to that of the material prepared from 2-aminobenzenenethiol, diethylaminopropyl chloride, and potassium hydroxide. The hydrochloride salt melted at 198–200° and gave no depression when mixed with authentic material.

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Physical Properties of Lipids Used in Pharmacy II

Use of Heating-Cooling Curves to Study Lipid Materials

By STUART P. ERIKSEN†, DAVIS R. REESE, and JOSEPH V. SWINTOSKY

This report describes the use of heating-cooling curves for the study of polymorphism as it occurs in chemically pure or commercial samples of glyceryl mono-, di-, and tristearate, theobroma oil, spermaceti, white petrolatum, and cetyl alcohol. The interpretation of the data obtained by the heating-cooling method has been simplified by using a log temperature difference plot. With this plot, the usual temperature-time curve can be rationalized into a series of interconnecting straight lines. The effects of heating or cooling rates on crystal form and crystal transition temperatures were studied with the aid of the improved heating-cooling curve plots. Distinct polymorphic transitions were observed in the pure glyceryl stearates. On the other hand, in commercial materials there was little evidence that the observed crystal changes were associated with specific polymorphic transformations. The relationship between the transitions observed and the probable storage stability (i.e., the maintenance of film integrity, moisture permeability, etc.) is discussed.

PHARMACISTS, in general, have not taken full advantage of the analytical techniques utilized in other fields for studying the crystal properties of lipids.[1] One purpose of our work was to determine how some of these techniques could be applied to the study of pharmaceutical products containing lipids. Part I of this series (1) presented a photomicrographic method for observing crystal changes and a recent note (2) has shown that polarized light transmission can also be a valuable technique for studying transitions in crystals. This report describes the use of heating-cooling curves for studying polymorphism as it occurs in pure and commercial samples of several lipid materials of pharmaceutical interest. In the heating-cooling curve technique, sample temperature is measured against time as the sample temperature changes in response to a change in environmental temperature.

The pharmaceutical uses of lipids as matrices, additives, coatings, etc., often demand a high degree of physical and chemical stability. To maintain both the acceptability and efficacy of lipid-containing products, such lipid properties as crystal size, refractive index, density, hardness, and film integrity must not change sufficiently during storage to impair the utility of the product. All of these properties are influenced by the crystalline state of the lipid at the time of testing or use.

The heating-cooling curve method is not the newest of the many analytical tools found useful in studies of the crystalline state but, because of its simplicity, it is still worthy of consideration, both as a research and a control procedure. Heating-cooling curves have proven their utility in the studies of chemically pure lipids (3, 4) and, because samples require minimum pretreatment, have also been found useful with commercial lipids for studying the effects of storage history. The studies on commercial lipids are more difficult to interpret, however, because the crystal changes are observed as small discontinuities on a logarithmic curve. The modification proposed in this report (i.e., replotting temperature vs. time data as logarithm temperature gradient vs. time) has been observed to simplify the graphic interpretation of heating and cooling processes (5a, b). With this modification, even minor changes in heat conduction or content appear as deviations from a straight line.

No attempt has been made in this report to document fully the literature of polymorphism, calorimetry, or even heating-cooling theory. A considerable body of recent information is already available in the literature in review form (3, 4).

EXPERIMENTAL

Raw Materials.—The cetyl alcohol was N.F. grade and purchased from M. Michel and Co. The white petrolatum, spermaceti, and theobroma oil were U.S.P. grade and purchased, respectively, from L. Sonneborn and Sons, McKesson and Robbins and Co., and A. N. Stollwerck, Inc. The commercial glyceryl monostearate was obtained from Hercules Chemical Co.; free glycerin 0.8%, monoglyceride content 36%. The commercial glyceryl distearate was obtained from Kessler Chemical Co.; free glycerin 0.6%, monoglyceride content 12%, saponification number 185, acid value 2.

Received April 28, 1961, from the Research and Development Division, Smith Kline & French Laboratories, Philadelphia 1, Pa.
Accepted for publication November 5, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
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[1] In this report, the term lipid is used in its broadest sense to include fats, waxes, and other related materials.

The synthetic glyceryl monostearate was prepared by the method of Fischer (6) from 1,2-glyceryl propylidene and octadecanoyl chloride. The saponification number of the purified product was 357.9 (theory 358.5).[2]

The synthetic 1,3-glyceryl distearate was prepared by the method of Jackson (7) from glyceryl monostearate, triphenylchloromethane, and octadecanoyl chloride.[3] The saponification number of the purified product was 311.9 (theory 312.5). The 1,3-structure was confirmed by infrared absorption.

The synthetic glyceryl tristearate was prepared by heating 1,3-distearin in benzene with octadecanoyl chloride and pyridine. The saponification number of the product was 296.1 (theory 297.2). Infrared analysis showed no unesterified hydroxyl groups.

Temperature Recording.—Using crushed ice as a reference bath, sample temperatures were measured with standard, cotton wrapped, 30-gauge iron-constantan thermocouples. The span of a Brown Electronik recorder was set to record temperature directly in °C. For heating curves, a thermoregulator-controlled 5-gal. water bath was used. For cooling curves, either room air or another 5-gal. water bath was used for cooling at or near room temperature, while a 4-L. Dewar flask filled with crushed ice was used for cooling to 0°.

Sample Cell.—Samples were contained in 10 × 75 mm. Pyrex test tubes, which were held in the special Pyrex cell shown in Fig. 1.

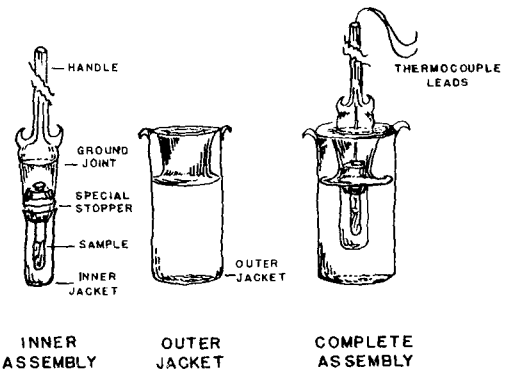


Fig. 1.—Pyrex cell for holding lipid samples during heating-cooling determinations.

Sample Preparation.—Solid samples are normally melted and cast directly into the sample tube. If prior history is being studied, however, the sample is cut into plugs which are then placed in the sample tube. Before measurement, a 2 × 100 mm. melting point capillary is set in the sample to serve as a thermocouple well and a small cork stopper is used to hold this capillary in the center of the test tube. Good contact between the sample and thermocouple capillary is important. Powdered material may be used if it is packed firmly around the capillary, but very coarse material was found to give poor results. Samples should be about 2 Gm. in size, but exact weight is not critical.

[2] The authors gratefully acknowledge the assistance of Dr. Robert J. Meyer of Morton Chemical Co., Woodstock, Ill., for the synthesis of the synthetic mono-, di-, and tristearins.

[3] The octadecanoyl chloride used was prepared from 97% stearic acid by fractional distillation of its methyl ester. By freezing point, its purity was estimated to be 99.9%.

Measurement Procedure.—The prepared test tube containing the sample is fitted into the inner assembly by using a double tapered rubber stopper (Fig. 1). The thermocouple is then inserted and the completed inner assembly is cooled to the starting temperature. (No heat transfer medium was used; the improvement so afforded did not warrant its use.) When the inner assembly has properly cooled, the outer jacket is attached, the complete cell immersed into a hot bath held about 5° above the melting point of the sample, and the temperature rise recorded. When the sample has melted, the cell is placed in the ice-filled Dewar flask until the cooling curve record indicates that the sample temperature is less than 20°, whereupon the sample is again placed in the hot bath and remelted. If desired, the effects of a smaller cooling gradient (i.e., slower cooling) may be studied by recooling the sample in a bath which is at either room temperature or 5° below the sample's freezing point.

Plotting Procedure.—The recorded curve (representing temperature in °C. vs. time) must be replotted as log temperature difference (bath temperature minus sample temperature) vs. time, or simply as log ΔT vs. time. By using 2-cycle semi-log paper and the procedure of Ball (5a), this replotting can be simplified. For heating curves, label the bottom ordinate as "bath temperature − 1°" then continue to label each ordinate upward in order,

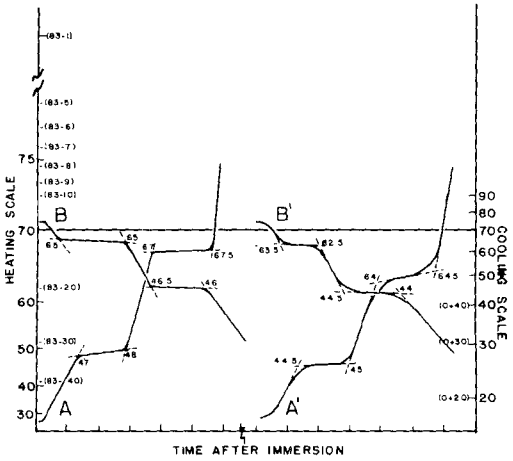


Fig. 2.—Log ΔT plots for a hypothetical substance. Curves A and B depict plausible heating and cooling curves, respectively, for a chemically pure substance. The addition of an impurity to this pure substance might alter the heating and cooling curves to those labeled A′ and B′, respectively. The labeling of the ordinates as described in the text is shown. In this labeling, the single number in the margin is the sample temperature. The first number within the parentheses is the bath temperature, while the second number is the difference between the bath temperature and the sample temperature. It is this latter (difference) number which controls the ordinate positions of the curves. The sample and bath temperatures are added only for convenience in plotting and reading the curves. Each curve is labeled as to (*a*) heating or cooling, (*b*) order of running (e.g., heating-1-, run before cooling-2-), and (*c*) the equilibrium bath temperature. Figures 3 to 13 were plotted in a manner similar to Fig. 2, but only the sample temperatures are designated on the ordinates.

bath temperature − 2°, *bt* − 3°, . . ., *bt* − 10°, *bt* − 20°, etc., until *bt* − *x*° reaches zero. Temperature vs. time is then plotted directly from the recorder curve using this temperature scale as the ordinate. For cooling curves, the bottom ordinate should be labeled "bath temperature + 1°" and the next *bt* + 2°, . . ., *bt* + 10°, *bt* + 20°, etc., and data plotted as before. The procedure described is shown in more detail in Fig. 2.

In the figures shown, all heating plots have been inverted to permit them to be studied in the same perspective as the cooling plots.

RESULTS AND DISCUSSION

Log Temperature Difference Plot.—The heating-cooling curves for chemically pure compounds are characterized by sharp inflections at the transition points. On the other hand, the curves of impure compounds, such as the usual commercial materials, have only minor inflections which are difficult to observe. To make the data on commercial materials easier to interpret, all heating and cooling curves were plotted as log (bath temperature minus sample temperature) vs. time. Called the log temperature difference plot (log ΔT plot), this temperature-time relationship has been found to be useful where samples are heated in constant temperature baths or ovens (5). In theory, as long as any crystalline form does not undergo change during heating or cooling, a log ΔT plot over this region will be a straight line whose slope is a function of the quantity of heat transferred and the specific heat of the crystalline form or forms present. Since phase transitions are accompanied by heat effects, however, the beginning or end of these will be indicated by inflections in the curves (3). Again, in theory, at the melting or transition point a straight line of different slope will be obtained as long as the coefficient of heat input remains fairly constant. These ideas suggest that a melting or solidification curve for a polymorphic material can be represented by a series of intersecting straight lines having different slopes as a result of the phase changes.

The degree of linearity of log ΔT plots is shown in Fig. 3 which depicts the heating and cooling of a substance (water) whose specific heat and phase

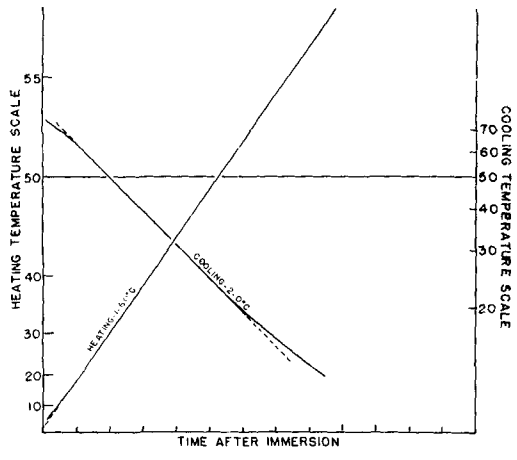


Fig. 3.—Log ΔT plots for the heating and cooling of distilled water.

structure remain the same throughout the temperature range studied. After an initial (10 minutes) equilibration period, in which a steady state heat input is being attained, the heating curve shows good linearity. The cooling curve shows a similar initial equilibration period with subsequent linearity until the sample temperature reaches 10–15°, where it deviates, probably because the ice bath was not stirred. Ball (5a) has observed that log ΔT plots are linear only until log ΔT = *ca.* 5° (i.e., until the sample temperature approaches to within 5° of the bath temperature). For this reason, the recording of heating-cooling curves is generally stopped when the sample temperature is within 2 or 3° of the bath temperature. This procedure limits the recording time in our studies to approximately 30 minutes for samples melting under 60°.

Heating and Cooling Studies of Nine Lipid Materials.—The log ΔT plots of heating-cooling curves for the nine lipid materials studied are shown in Figs. 4–13. The heating ordinates on these graphs are on the left, while the cooling ordinates are on the right. Because the scales cannot be made the same (the heating scale is an inverted log function, while the cooling scale is a normal log ordinate), some compromise had to be made in order to plot heating and cooling on the same graph. One temperature near the center of both curves was used as a point of correlation and the rest of the values were graphed from it (see 50° line, Fig. 3). Only at this temperature are both scales exactly the

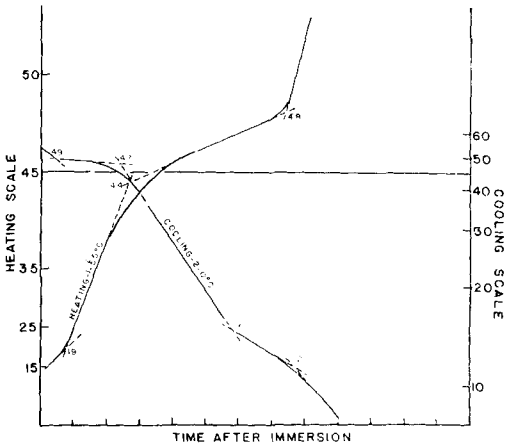


Fig. 4.—Log ΔT plots for the heating and cooling of cetyl alcohol.

same. For clarity, the temperatures of all inflection points have been noted directly on the graphs (e.g., 47 and 48° on curve A of Fig. 2). Each curve is labeled as to (*a*) heating or cooling, (*b*) order of running (e.g., heating-1-, run before cooling-2-, run before heating-3-, etc.), and (*c*) the equilibrium bath temperature.

To clarify interpretation of the data, heating-cooling curves of a hypothetical sample are presented in Fig. 2, preceding a discussion of the data of the lipid samples. In Fig. 2, curves A and B might be the heating and cooling curves, respectively, of a pure substance, while A′ and B′ might be the corresponding curves for the same substance in a less pure state. For simplicity, the same transition is

shown in all four curves, i.e., a reversible (enantiotropic) transition at approximately 47° in the pure substance (curve A). Transition temperatures are often lower on cooling curves because of a tendency to supercool. All four curves exhibit straight line segments, but the slope changes are more abrupt with the pure substance. At the beginning of each curve, there is a time lag before steady state heat transfer occurs across the cell. Most transitions in the lipid substances occur over a temperature range, e.g., in curve A, the transition starts at 47° and ends at 48°. To get exact transition temperatures, it is usually necessary to extrapolate straight line segments until they intersect, e.g., 44.5° on curve A

The terminology used in the following discussion is that found in the literature (3, 4). In general, the forms are named gamma (γ), alpha (α), beta prime (β'), and beta (β), in order of increasing melting points. The β form is the stable form in the materials reported, but in rare cases it is not known to exist and the β' is the stable form.

Figures 4, 5, and 6 show the heating and cooling curves of commercial samples of cetyl alcohol, white petrolatum, and spermaceti. These same N.F. and U.S.P. materials have been studied dilatometrically by Ravin and Higuchi (8). Cetyl alcohol shows a smooth heating curve with a break at 19° and a melting range (44–48°) which ends sharply at 48°.[4] The inflection at 19° probably represents the melting of a component of the commercial material which is normally liquid at room temperature. On the cooling curve, this

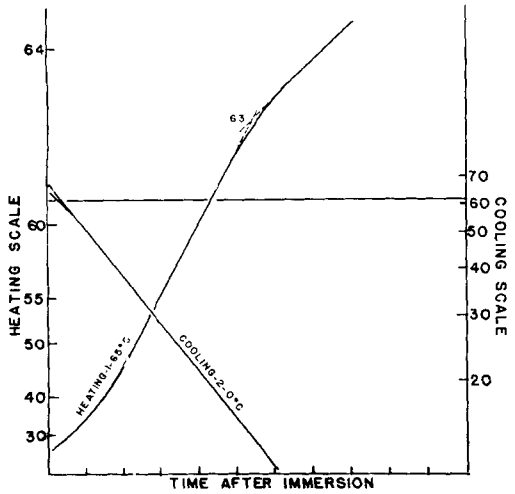


Fig. 5.—Log ΔT plots for the heating and cooling of white petrolatum.

transition occurs between 15 and 12° rather than at 19°. An inflection at 40–45°, observed by Ravin and Higuchi, could not be observed in our studies. In contrast, since they did not study cetyl alcohol below room temperature, they did not report the transition at 19°. Both white petrolatum (Fig. 5) and spermaceti (Fig. 6) show smooth, uninterrupted approaches to the melting point, i.e., there are no detectable transition points. The results are

[4] It is generally a misnomer to speak of melting points in lipids. Any sensitive method will detect a melting range.

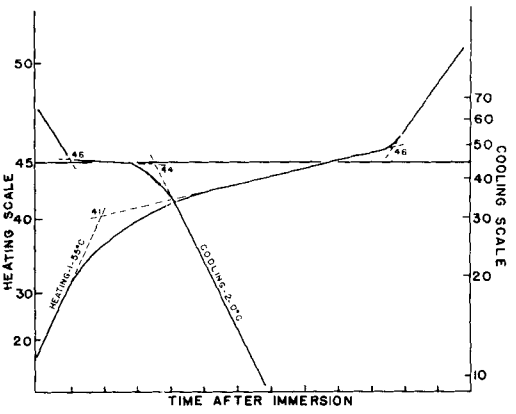


Fig. 6.—Log ΔT plots for the heating and cooling of spermaceti.

essentially the same as those obtained dilatometrically.

Heating and cooling curves run on synthetic glyceryl monostearate (monostearin) are shown in Fig. 7. This material is reported to exist in four forms; namely sub-alpha, alpha, beta prime, and beta (4). The curve labeled heating-1- is the heating curve of material stabilized by prolonged storage at room temperature. Two distinct inflections (65–68° and 74–76°) can be observed and, although they are 5° less than those reported by Malkin (9a), they probably represent the α (along with the subsequent $\alpha \rightarrow \beta$ transition), and the β melting points. The β' form is difficult to observe by these methods (9b). A heating curve, heating-3-, run after rapid cooling (i.e., there was a large cooling gradient), shows only melting of α form crystals, no β form being present or formed during the heating. There is an indication on curve heating-3- of an arrest at 39°, but it is too indefinite to be conclusive. Slow cooling (i.e., using a small cooling gradient) yields essentially the same results except for a definite arrest at 47°, which probably represents alpha → sub-alpha transition (see curve cooling-4-). Commercial

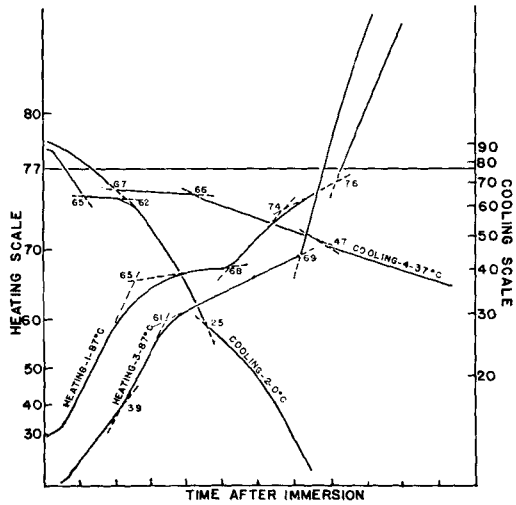


Fig. 7.—Log ΔT plots for a heating-cooling study of synthetic glyceryl monostearate.

monostearin shows little, if any, evidence of polymorphic change when it is remelted after being rapidly cooled (see curves cooling-2- and heating-3- on Fig. 8). While polymorphic transitions probably do occur in commercial monostearin, the presence of large amounts of other glycerides (palmitates, oleates, etc.) and/or other substitutions (di- and triester) apparently obscures the heat effects of these changes.

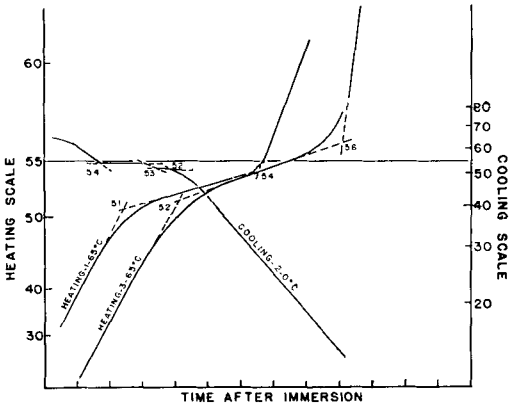


Fig. 8.—Log ΔT plots for a heating-cooling study of commercial glyceryl monostearate.

The heating and cooling behavior of the synthetic glyceryl distearate (distearin) is shown in Figure 9. Forms reported in the literature are α, β', and β (4). Clearcut evidence of two crystal forms appears in the heating curve run on a sample cooled rapidly from a melt (see heating-3-) with the transition occurring at 57–60°. These two forms correspond roughly to the α and β' forms reported by Baur, *et al.* (10a), and Malkin and Shurbagy (10b). The β' melting observed on reheating (heating-3-) could have resulted either from rapid transition of α to β' as the heating curve was being run or from β' crystals formed during the cooling. The curve obtained during rapid cooling (cooling-2-) suggests that very little β' form crystallizes out with the α form. At slower cooling rates, however, both forms definitely appear (cooling-4-).

Note that cooling curves normally produce freezing points well below the melting points of the same form. Smit (11) has theoretically explained this as due to the kinetic nature of heating and cooling determinations, and suggests that only heating curves be used for exact work.

For the same reasons as indicated above with commercial monostearin, the heating-cooling curves of commercial distearin do not show the polymorphic forms found in the pure material (Fig. 10). As expected, the melting and freezing points of this commercial distearin are considerably below those of the synthetic material. The melting point of the commercial mixture is lowered by rapidly cooling the melt (compare heating-3- with heating-1-), but none of the three curves indicates a characteristic polymorphic transition. Lutton (12) has suggested that the higher melting points of aged samples (also observed in synthetic distearin) are due to crystal aging or stabilization. This point is discussed further under theobroma oil.

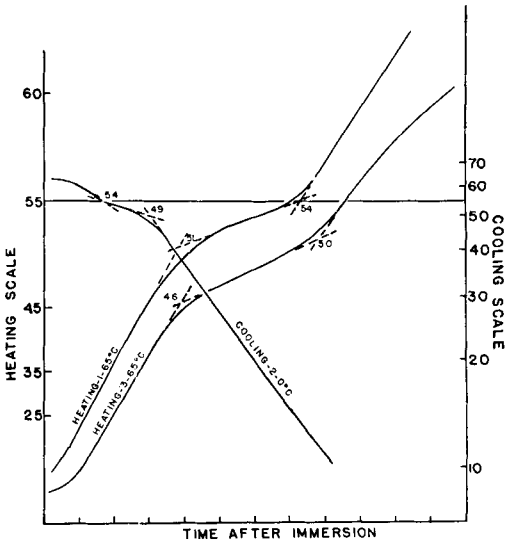


Fig. 10.—Log ΔT plots for a heating-cooling study of commercial glyceryl distearate.

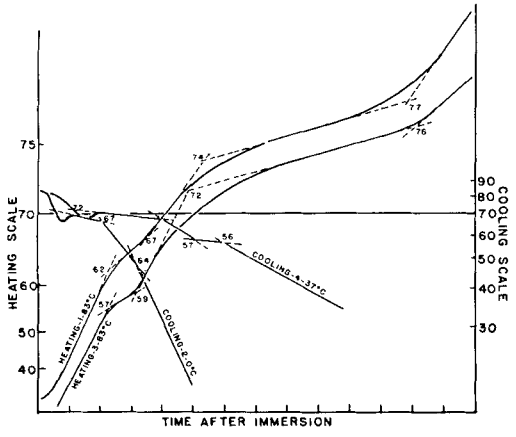


Fig. 9.—Log ΔT plots for a heating-cooling study of synthetic glyceryl distearate.

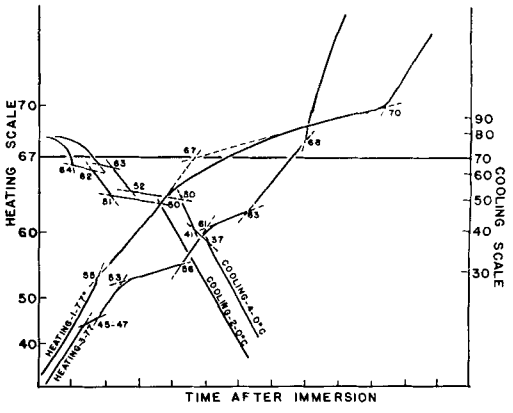


Fig. 11.—Log ΔT plots for a heating-cooling study of synthetic glyceryl tristearate.

Rapidly cooled synthetic glyceryl tristearate shows four distinct melting or transition points, while storage stabilized samples show one or perhaps two (Fig. 11, curves heating-3- and heating-1-, respectively). Three of these points correspond well to the α, β', and β melting points reported by Clarkson and Malkin (13) (melting points found 53–56°, 61–63°, 67–70°; reported in the literature 55°, 65°, 72°). The lowest form observed in this work, melting at 45–47°, is not identifiable from the literature.

Theobroma oil (cocoa butter), although a mixture of several glycerides, has a heating-cooling curve similar to that of a pure compound. This has been explained (14–16) as being due to the high percentage (more than 50%) of one glyceride, 2-oleopalmitostearin. The forms γ, α, β', and β melting at 16–18°, 21–24°, 27–29°, and 34–35° have been reported (17, 18). The heating-cooling characteristics of a commercial sample of theobroma oil are shown in Figure 12. The storage stabilized sample (heating-1-) shows essentially one melting point (33°), that of the stable β form. Although this curve shows no indication of β' crystals (m.p. 28°), some are probably present because both β' and β forms are quite stable at room temperature (17a). Cooling molten theobroma oil to 0°

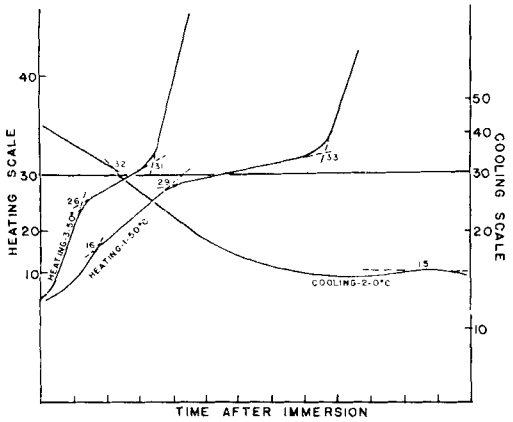


Fig. 12.—Log ΔT plots for a heating-cooling study of a storage stabilized sample of theobroma oil (cocoa butter). Curve cooling-2- was run within 2 hours of curve heating-1-. Curve heating-3- was obtained after the sample used for curve cooling-2- had stood at room temperature for 24 hours.

results in a congealing point of 15–16°. From its translucent appearance, this is probably a glassy form, although Vaeck describes it as the γ form (17c). If this glassy form is immediately reheated, it melts at 16–25° (see, for example, heating-1-, Fig. 13). On the other hand, if the glassy form is allowed to stand for 24 hours at room temperature, complete transformation occurs, primarily to the α form, but at least in part to the β form. If the sample is now reheated (see heating-3-, Figs. 12 and 13) the influence of the β nuclei can be observed; complete melting of the sample occurs above the β' melting point (i.e., >28°).

If theobroma oil (cocoa butter) suppositories are prepared by standard suppository procedures (melt at 40°, pour into 0–2° mold), the suppositories, when immediately reheated, exhibit the behavior shown in heating-1- of Fig. 13. Only $\gamma \rightarrow \alpha$ transition is observed, plus α melting at 25°. When the suppositories are allowed to stand at room temperature for 24 hours after casting, the heating curve (heating-3-) shows that complete transition to the α form and some $\alpha \rightarrow \beta$ transition has occurred. It is unlikely that $\alpha \rightarrow \beta'$ transition occurs in studies conducted under the described conditions because this transition is quite slow when compared to the $\alpha \rightarrow \beta$ transition (17b).

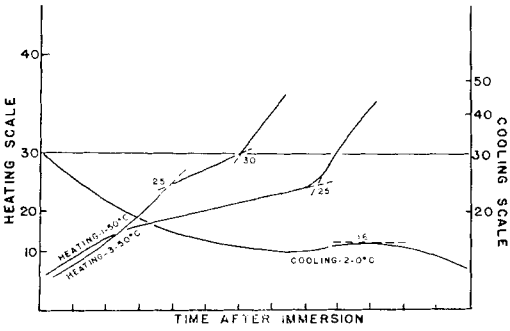


Fig. 13.—Log ΔT plots for a heating-cooling study of theobroma oil (cocoa butter) suppositories (cast at 0 to 2° from a 40° melt). Curves heating-1- and cooling-2- were obtained within 2 hours of casting. Curve heating-3- was obtained after the suppository had stood at room temperature for 24 hours.

In most of the materials where an aged sample was compared with a recently crystallized sample (Figs. 7–13), the melting point of the recently crystallized sample was found to be lower. This has been discussed by Steiner (18) who suggests that these low melting points are due to "mutually soluble binary mixtures." In light of the observation that rapid cooling also produces low melting points, the simpler explanation suggested by Lutton (12), that all samples which are not completely "aged" will give low melting points (lower by as much as 3°), seems a more acceptable one.

Stability Interpretations of Heating-Cooling Data.—Polymorphism is defined as the ability of a substance to exist in more than one crystal form. These crystal forms have different physical properties, e.g., melting points and different energy contents. They are of importance because transitions from one crystal form to another, with simultaneous changes in physical properties, can cause stability problems in pharmaceutical products even under room temperature storage conditions.

Essentially two large classes of polymorphic transitions have been identified in lipids. In the more common type, called monotropism, one crystal form is stable up to the melting temperature. Under these conditions, metastable crystals will tend to transform, with time, to the stable form, irrespective of storage temperature; however, the storage temperature may influence the rate of monotropic transitions. In the less common type of polymorphism, called enantiotropism, each crystal form has a temperature range in which it is stable, and reversible transformation occurs between two forms at the transition temperature.

Because the main concern in lipid stability is the presence and rate of polymorphic transitions rather than their direction, both types of polymorphism may be discussed together.[5] The transition from one polymorphic form to another, with its consequent liberation of heat (heat of transition), probably occurs as both a $solid_1 \rightarrow solid_2$ and a $solid_1 \rightarrow liquid \rightarrow solid_2$ phenomenon (11, 13). The extreme slowness of known $solid_1 \rightarrow solid_2$ transitions (e.g., methyl stearate and palmitate) (19), along with the sharpness and reproducibility of lipid transition temperatures, suggest that $solid_1 \rightarrow liquid \rightarrow solid_2$ transition is the predominant mechanism for transitions occurring at temperatures near the maximum transition temperature. At temperatures below this temperature, some much slower mechanism may be responsible for the crystal transitions (e.g., $solid_1 \rightarrow solid_2$). Since solid phase transitions would logically be functions of viscosity, transformations which have high transition temperatures will probably proceed at negligible rates at room temperature in solids which have a low liquid content. Complete transition, therefore, may require minutes, days, or years, depending on the transition rate at the temperatures of storage.

Estimation of a lipid material's potential for polymorphic change, or in other words its tendency to be unstable, can be made by comparing the heating curve of a sample stored for prolonged periods at room temperature (i. e., stabilized) with one of a sample which has recently been rapidly frozen from a melt. Freezing from a melt usually produces the least stable (γ and/or α) forms of lipids, and reheating the sample will then show the presence of these forms. If a stabilized sample of material is not available, the observation of multiple melting points in a rapidly-frozen sample may be used as criteria of polymorphic potential.

All of the synthetic stearates studied show great polymorphic potential. Synthetic glyceryl monostearate (Fig. 7), glyceryl distearate (Fig. 9), and glyceryl tristearate (Fig. 11), all show multiple melting points even after prolonged storage at room temperature. The degree of transition to the stable polymorphic form was so great in the di- and tristearates, however, that considerable information would have been lost if the rapidly-cooled samples had not been reheated. The commercial stearates (Figs. 8 and 10) show no such polymorphic potential. The difference in melting points observed on the two heating curves of commercial distearin is related to the difference in the "ages" of the two samples, as was discussed in a previous section. It was also mentioned previously that the absence of distinct transition points in commercial materials should not be interpreted to mean that polymorphism is absent. Chemical heterogeneity of a given sample tends to obscure its polymorphic transitions. Careful studies, including the use of purified components of the sample, may be necessary to prove their existence.

The real value of heating-cooling curves, from a stability point of view, is in estimation of the relative rate of a polymorphic transition at room or storage temperature. With lipid-containing pharmaceuticals, we are concerned only with a rather narrow range of transition rates and can adjust the parameters of the heating-cooling measurement procedure so that these rates will be observed. On the one hand, if the transition rates are so high that low melting forms are not observable by heating-cooling methods, the crystal changes in the lipid will probably be completed during the manufacturing process and cause no stability problems in the product. On the other hand, if the crystal changes are extremely slow at the transition temperature or if the transition temperatures are much higher than the storage or use conditions, the rate of transition at room temperature may be negligible and, again, there probably will be no stability problems. Therefore, it seems likely that stability problems occur in pharmaceutical products only when the transitions proceed at rates between these extremes under the temperature conditions to which the product is exposed.

If one considers the synthetic stearin series (Figs. 7, 9, and 11) and in particular the transitions in the range 58–68° (monostearin, $\alpha \rightarrow \beta$; distearin, $\alpha \rightarrow \beta'$; tristearin $\beta' \rightarrow \beta$) the relative rates of these transformations can be estimated. At the transition temperature (61–69°) monostearin showed no transformation to the β form (no 76° m.p.), tristearin (at 61–63°) transformed very slightly (slight melting at 68°), and distearin rather completely. One could expect that the polymorphic stability of these pure lipids would decrease in a similar order (i.e., stability of monostearin > tristearin > distearin). At room temperature, the absolute rate of these transitions would also be influenced by the transition temperature itself. The higher transition temperatures of mono- and tristearin would further suggest that their room temperature transition rates would be less than that for distearin. Another example of this type is shown in theobroma oil, which has the same slow $\alpha \rightarrow \beta$ transition as was shown by rapidly cooled mono- or tristearin (e.g., see Fig. 13, heating-1-). The room temperature transition rate of theobroma oil is fairly rapid (heating-3-) because the transition temperature is near room temperature.

Another possible use for studies like those illustrated here concerns the effects of heat treatment (tempering) on the crystalline state of the lipid. Crystal transformation could be monitored easily by storing the material at the desired temperature and observing the effect of storage on the heating curve of the stored material. Tempering near the transition temperature would then continue until the undesired form had disappeared from the curve.

In general, our experience suggests that the smoother and straighter the entire log ΔT vs. time plot of a heating-cooling curve, the greater the crystal stability of a lipid. Secondarily, the higher the transition temperature, the greater the crystal stability at room temperature.

Effects of Polymorphic Forms on Lipid Properties. —A basic characteristic of polymorphic transitions is the decrease in specific volume which normally occurs as the crystalline form changes from $\gamma \rightarrow \alpha \rightarrow \beta' \rightarrow \beta$. This decrease in specific volume, if it occurs after a container has been filled or a lipid film has been applied, can cause shrinkage away from the container walls or loss in integrity of the film. Changes in the crystal form can also cause more subtle changes, such as in the vapor and

[5] If the storage conditions of stability testing include temperatures above and below the enantiotropic transition temperature, this assumption is not necessarily true.

liquid permeability of a film. It has been observed, for instance, that rapidly cooled wax films transmit water vapor much faster than stabilized films (20, 21), which suggests that crystal size, shape, form, and orientation may have a pronounced effect on vapor and liquid transmission.

Pharmaceutical Utility of Heating-Cooling Curve Techniques.—It has been shown that most fats and waxes are polymorphic and on aging may undergo crystalline changes (3). Since these changes sometimes occur over a significant time span they can cause physical changes in pharmaceutical products during storage, e.g., changes in ointment consistency, changes in the appearance of suppositories, or changes in the integrity of tablets, pellets, or other dosage forms containing lipids. The development pharmacist needs good methods for determining what lipids are likely to undergo such changes, the temperatures at which the changes are accelerated, the rates at which they occur, and methods for eliminating or minimizing these changes during or after the manufacture of dosage forms which contain lipids.

Heating-cooling curves are useful for detecting the propensity of these pharmaceutical agents to undergo polymorphic change, for determining the temperatures of rapid polymorphic transitions, for evaluating the effects of time-temperature history on the polymorphic form which a lipid may assume, and for establishing what manufacturing and handling procedures result in the unstable or the relatively stable crystalline forms.

If, for example, heating-cooling curve data indicate that a substance undergoes polymorphic change in a pharmaceutical product at the usual temperatures of storage and transit, the pharmacist must be alert to the fact that a stability problem may result. Specific manufacturing conditions may be necessary to assure that the integrity of the product remains unchanged from lot to lot. Products containing cocoa butter, for example, fall into this category and may show different degrees of polymorphic change and different physical stabilities, depending upon their methods of preparation, despite the fact that their chemical compositions may be identical.

In other instances, polymorphic changes may be completely inimical to the purpose for which the product is intended. In such instances a stable form must be attained when the product is initially prepared. This can either be accomplished through the use of tempering or special manufacturing techniques. More simply, the lipids may be chosen on the basis of their inabilities to undergo significant polymorphic changes in the product formulas selected and under the anticipated storage conditions. In all product development problems involving lipids, the heating-cooling curve technique can be of considerable value in evaluating the raw lipid materials and as a control method for establishing the materials and manufacturing procedures necessary for attaining the required physical integrity.

The heating-cooling curve measurements described in this study require only relatively simple apparatus, are inexpensive to perform, and can be performed by unskilled personnel with a minimum of training. The quantity of sample required is only about 1 or 2 Gm.; however, in some instances this might be considered inordinately large, as for example when evaluation of a pellet or tablet film is involved. In such an instance, the light transmission technique (2), which utilizes samples weighing only a few mg., might be a preferred evaluation technique.

Because the heating-cooling curve technique of this study is a nonequilibrium method, it is much more rapid than the dilatometric technique (8). A number of samples may be run in a relatively short period of time. On the other hand, it gives no direct indication of the volume changes involved when polymorphic transitions occur. When volume change data are important, the dilatometric technique is valuable, and the heating-cooling approach of this study is of little use unless a direct correlation can be found between the two methods with the particular system under study.

Within the limitations discussed, the heating-cooling curve technique has value in studies of polymorphism. It represents an approach which could see greater use in the solution of pharmaceutical problems relating particularly to the use of lipids in dosage forms.

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Defibrillatory Substance from *Pteryxia terebinthina*

By GORDON H. BRYAN

A method for the isolation of a crystalline material from the roots of *Pteryxia terebinthina* has been described. The crystalline material was found to be essentially free from contractile depressant effects, and to be approximately seven times more potent than quinidine sulfate as a defibrillatory agent on isolated rat hearts.

A DESCRIPTION and the history of *Pteryxia terebinthina* (Hook.) Coult. & Rose, var. *terebinthina*, has been written by Pettinato (1) and Call (2). Call (2) obtained a crystalline material that he named pteryxin from the powdered root of this plant. A preliminary chemical and physical characterization was made by Pettinato (1) and later extended by Willette and Soine (3). Pteryxin was reported as having uterine relaxant and coronary vasodilator properties comparable to those of khellin (2, 3).

In unpublished experiments by the author crude, dealcoholized extracts of the root also demonstrated defibrillatory properties and, for this reason, it was of interest to isolate the active principle and compare its cardiac effects with those of quinidine.

EXPERIMENTAL

Extraction and Isolation

A June collection of the root was obtained from the vicinity of Moses Lake, Washington. The roots were dried and powdered in the same manner as described by Pettinato (1). A 5-Kg. quantity of the powder was macerated for 12 hours with 80% ethyl alcohol and extracted by percolation with approximately 60 L. of the menstruum. The percolate was evaporated by forced air draft until an aqueous and oily layer had separated. The aqueous layer was decanted, treated with a saturated lead subacetate solution, and filtered until no further precipitation occurred. Hydrogen sulfide was then passed through the filtrate. Excess lead, as the sulfide, was removed by filtration. The filtrate was evaporated by air to a thick syrupy consistency and partially redissolved in 95% alcohol. Insoluble material was removed by filtration. The clear liquid was stored at room temperature with access to air. Crystals appeared in the bottom of the container after 3 to 4 days. The supernatant fluid was decanted and the crystalline material redissolved in acetone with the aid of a small amount of distilled water. Partial evaporation at room temperature of this solution resulted in recrystallization of the material. Approximately 2 Gm. of crystals were obtained in this manner.

The crystals melted between 191–192°. They were very soluble in water and the solution had a weak blue fluorescence that was not apparently altered by the addition of dilute sulfuric acid nor by dilute ammonium hydroxide. No further attempt was made to determine physical or chemical characteristics.

Pharmacological Studies

Isolated Rat Heart.—Because of availability and small size, rat hearts were selected for this investigation. The procedure and apparatus for left intraventricular cannulation and coronary perfusion as described by Bryan (4) was followed. The composition of the perfusing fluid was: (Gm./L. distilled water) NaCl 7.0, KCl 0.4, $CaCl_2 \cdot 2H_2O$ 0.4, $MgCl_2 \cdot 6H_2O$ 0.2, Na_2SO_4 0.14, $NaHCO_3$ 1.5, and dextrose 1.0. The fluid was gassed with oxygen to saturation and the pH adjusted to 7.4 by means of a Beckman (model H-2) pH meter and with 5% carbon dioxide in oxygen. Perfusion pressure was approximately 40 mm. Hg and at a temperature of 37°.

Quinidine sulfate,[1] aconitine nitrate,[2] and the crystalline material that was isolated were dissolved separately in perfusion fluid for injection into the perfusion apparatus directly above the aortic cannula. Stock test solutions were prepared the day they were to be used.

Surface left ventricular electrogram was recorded through a wick electrode placed on the body at the left ventricle. Left ventricular pulse pressure (via strain gauge) was recorded with the electrogram by means of a two-channel Sanborn apparatus.

Cardioplegia.—Preliminary quantitative analysis with quinidine sulfate indicated an approximately linear relationship between dose and pulse pressure reduction that terminated in reversible cardioplegia. Prior to the isolation of the crystalline material, crude extracts of *terebinthina* also had this effect. Therefore, cardioplegia was the depressant effect used for a comparison of the two substances.

In order to test contractile depression independently from rate, hearts were driven by a Grass square wave stimulator. Rate was constant for each heart but varied between hearts.

Doses from a starting value of 100 mcg. of quinidine sulfate were increased by alternate arithmetic and geometric progression until cardioplegia was achieved. A comparable dosage schedule for the crystalline material was abandoned because of the rapid depletion of the supply, and an unexpected finding.

Received April 28, 1961, from the School of Pharmacy, Montana State University, Missoula.

Accepted for publication December 26, 1961.

This work was supported in part by a Sterling-Winthrop research grant.

Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

The author wishes to acknowledge indebtedness to T. G. Call for identification and samples of *Pteryxia terebinthina*, R. L. Van Horne for providing unexpected necessities, J. L. Wailes for interest and aid, and F. A. Pettinato for helpful suggestions.

[1] Merck & Co., Inc., Rahway, N. J.

[2] S. B. Penick & Co., New York, N. Y.

Fifteen hearts required an average ± S.D. of 1.72 ± 1.4 mg. of quinidine sulfate to obtain cardioplegia. In contrast, of 10 hearts, 4 showed a pulse pressure reduction of approximately 50% and 6 did not show an obvious contractile change at a dosage of 50 mg. of the crystalline material. Cardioplegia did not occur in two additional hearts with 100 mg. of the crystalline material. No further attempt was made to produce this effect and it was concluded that the crystalline material was virtually without a contractile depressant action on the rat heart.

Fibrillation.—Spontaneously beating rat hearts were used for this portion of the investigation. In order to cause fibrillation, 40 mcg. of aconitine nitrate was injected into the perfusing system. Ventricular fibrillation occurred within 1 minute, however, spontaneous reversion also occurred within approximately 5 minutes (10 hearts). Inasmuch as this unexplained circumstance was not useful, the relevance of potassium reduction in the perfusing fluid as determined by Kärki (5) was explored.

When the potassium chloride content of the perfusing fluid was reduced one-half by isosmotic sucrose substitution, aconitine-induced fibrillation was considered persistent if it lasted 15 minutes and longer. This was found to occur without exception for 10 hearts and is in agreement with Kärki whose observations were made on rate-induced fibrillation with isolated rabbit hearts. Accordingly, hearts fibrillating for 15 minutes after aconitine injection at a reduced potassium perfusing concentration were used to test defibrillation action of quinidine and the crystalline material.

Starting doses of 100 mcg. with arithmetic progression were used for quinidine sulfate, and a similar procedure starting at 10 mcg. was used for the crystalline material. To defibrillate 20/20 hearts required an average ±S.D. of 350 ± 36 mcg. of quinidine sulfate and 50.0 ± 9 mcg. of the crystalline material. At a $P = 0.05$, these means are significantly different. Therefore, it can be stated that the crystalline material possesses a potency of approximately seven times that of quinidine sulfate in its ability to defibrillate rat hearts.

Pulse pressures (reduced to essentially zero after fibrillation) were not restored to control values after defibrillation by either agent. In view of the return to control values when perfusion is continued after quinidine-induced cardioplegia, this observation was of interest. Whether aconitine or low potassium is a causative factor is not known.

In addition, when hearts in either series were defibrillated, fibrillation could not be reproduced by aconitine. This suggests a comparable, although unknown, mechanism of action for quinidine and the crystalline material.

The rat heart was selected, in part, on the premise that smaller quantities of test chemicals would be required, and it was of interest to note that the average quinidine defibrillation dose was nearly the same as that required for this purpose by isolated rabbit hearts (6). This would indicate that concentration rather than absolute amount is a critical factor in defibrillation under these conditions.

DISCUSSION

If the mechanisms for electrical and mechanical activity are considered as separate, but interrelated, then it is of obvious value to select, for defibrillatory purposes, those chemicals that have a higher degree of specificity for the former but with little or no effect on the latter. On this basis, the crystalline material isolated from *Pteryxia terebinthina* would then be preferred to quinidine. However, there are a number of other drugs and chemicals available for this purpose. Information regarding cardioplegic/defibrillatory ratios for these substances is not readily available. Therefore, no further conclusions can be reached concerning the relative potency of the crystalline material.

A second consideration—extensive information regarding *in vivo* effects of the crystalline material was not obtained. This was because of the limited quantities of the crystalline material available. In order to overcome this handicap, an attempt is being made to determine the chemical identity of the crystalline material with synthesis as an eventual objective.

CONCLUSIONS

1. A procedure for the isolation of a crystalline material from *Pteryxia terebinthina* has been described.
2. Through comparison on the isolated rat heart, the crystalline material has been shown to have a defibrillatory potency approximately seven times that of quinidine sulfate.
3. In contrast to quinidine, the crystalline material does not produce cardioplegia at a dose level of approximately 30 times that of quinidine sulfate.
4. A procedure for obtaining persistent fibrillation with aconitine in the isolated rat heart has been described.

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Paper Chromatographic Determination of Muscarine in *Inocybe* Species

By J. K. BROWN, M. H. MALONE†, D. E. STUNTZ, and V. E. TYLER, Jr.

A relatively simple, paper chromatographic method was developed for the quantitation of muscarine in dried mushroom tissue. Application of the procedure to 34 species of *Inocybe* revealed detectable quantities of muscarine ranging from 0.01 to 0.80% in approximately 75% of them. No significant relationship was found between distribution of muscarine and infrageneric taxonomy of the genus *Inocybe*.

THE GENUS *Inocybe* is composed of nondescript, generally brownish agarics, often with characteristic odors. Although recognition of a mushroom as an *Inocybe* is comparatively simple, species identification is extremely difficult, even when microscopic characteristics are considered. In 1924, Kauffman (1) recognized 105 North American species, but today this number is known to be very conservative. Unfortunately, no modern comprehensive treatment of domestic species is available; the best European monograph is that of Heim (2).

Not all species of *Inocybe* are toxic, but muscarine is the toxic principle found in all poisonous species which have been examined. Eugster (3) has reviewed the older literature on the occurrence of this compound in mushrooms and has pointed out that the concentrations reported by Loup (4) for *Inocybe* species are too high, being based on physiological tests compared with an impure muscarine standard. Isolation of muscarine from several European species of *Inocybe* has recently been carried out (5). Percentage concentrations, calculated on fresh-weight basis, have been reported for the following species (*sic*): *I. fastigiata* (Fr. ex Sch.) Quél., 0.01%; *I. patouillardi* (Bres.), 0.37%; and *I. umbrina* (Bres.), 0.003%.

Species of *Inocybe* are abundant in western Washington and pose an appreciable health hazard to the indiscriminate collector. It was of interest, therefore, to develop a procedure which would permit the rapid screening of small quantities of carpophore tissue in order to determine the relative toxicity of the species.

Received January 17, 1962, from the College of Pharmacy and the Department of Botany, University of Washington, Seattle 5, and the Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs.

Accepted for publication February 21, 1962.

Abstracted from a dissertation submitted to the Graduate School of the University of Washington by J. K. Brown in partial fulfillment of the requirements for the M.S. degree.

This investigation was supported by research grants No. RG-7515 (University of Washington) and RG-8239 (University of Connecticut) from the National Institutes of Health, Bethesda, Md.

The authors are indebted to Dr. R. G. Benedict and Dr. L. R. Brady for assistance in the collection of the *Inocybe* species employed in this investigation, to Mrs. G. O. Kempton for supplying the collection of *I. nigrescens* from Alaska, and to Dr. P. H. List for a generous sample of pure muscarine gold chloride.

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EXPERIMENTAL

Inocybe Species Examined.—The naturally occurring carpophores used in this investigation were collected in western Washington during the fall and early winter of 1960 through the spring of 1961. One collection of *I. nigrescens* was obtained from the Portage Glacier area in Alaska. Representative herbarium specimens of each collection were deposited in the mycological herbarium of the University of Washington. Some of the species investigated have not been described in the literature; consequently, these are referred to by a herbarium number pending publication of their characteristics.

All collections from western Washington (Table I) were cleaned, dried in a forced-air drying oven at 48° for at least 72 hours, and stored in tightly closed glass containers until examined. The species of Alaskan origin which had been dried prior to shipment was redried and treated similarly. Each collection of any given species was maintained separately from other collections of the same species obtained at other times or places.

Extraction and Purification Procedures.—A number of different methods were investigated to determine their suitability for the quantitative extraction of quaternary ammonium compounds from the dried carpophores. Homogenization with 70% ethanol in a Waring Blendor, extraction of finely powdered carpophores by shaking for prolonged periods with 70% ethanol, and continuous extraction of finely powdered carpophores in a Soxhlet apparatus with 95% ethanol proved to be relatively inefficient extraction procedures. When carpophores (5.0 Gm.) were milled to a No. 40 powder, defatted, macerated for 1 hour with a mixture of absolute ethanol:10% ammonium hydroxide solution (19:1), and extracted in a Soxhlet apparatus with the same solvent for 33 hours, chromatographic examination of a subsequent extract of the marc failed to detect residual quaternary compounds.

Purification of the extract was effected by concentrating it under reduced pressure at 50° in a flash-evaporator until a thick syrupy residue (approximately 2 ml.) was obtained. This residue was dissolved in successive small volumes of distilled water which were combined (total 25 ml.). Sufficient sodium hydroxide was added to produce a concentration of 3 *N*, and 5.0 ml. of a 13% solution of ammonium reineckate in methanol was added to this alkaline solution. The mixture was then refrigerated at 5° for 10 to 12 hours, centrifuged, the supernatant liquid decanted, and the precipitate washed with cold *n*-propanol (three 2-ml. quantities) until the washings were colorless. The washed precipitate was then dissolved in successive small

TABLE I.—TIME AND PLACE OF COLLECTION OF THE SPECIES INVESTIGATED

Species of *Inocybe*[a]	Collection No.	Area of Collection (Washington)	Date
picrosma	FHPi-1	Friday Habor	11-6-60
1790[b]	UWP-1	U. of W. Campus[c]	12-1-60
1838	FHG-1	Friday Harbor	11-6-60
terrifera	OT-1	Olympia	5-20-61
geophylla	FHGG-1	Friday Harbor	11-6-60
lilacina	FHL-1	Friday Harbor	11-6-60
pudica	LPPu-1	Lincoln Park[c]	12-2-60
pudica	OP-1	Olympia	10-23-60
agglutinata	FHAg-1	Friday Harbor	11-6-60
3691	FHH-1	Friday Harbor	11-6-60
subdestricta	LFS-1	Lee Forest[d]	9-21-60
2147	OSu-1	Olympia	10-23-60
2147	A2147-1	U. of W. Arboretum[c]	11-14-60
3365	TSu-2	Tenino	4-1-61
griseolilacina	FHGr-1	Friday Harbor	11-12-60
3399	FHNo-1	Friday Harbor	11-12-60
3761	TO-1	Tenino	11-20-60
3761	OO-1	Olympia	10-27-60
2907	FHPo-1	Friday Harbor	11-6-60
1774	FHNo-2	Friday Harbor	11-12-60
3983	LPPg-1	Lincoln Park	11-2-60
lacera	TL-1	Tenino	11-20-60
lacera	FHLa-1	Friday Harbor	11-12-60
griseoscabrosa	FHGs-1	Friday Harbor	11-12-60
pallidipes	LPP-1	Lincoln Park	11-21-60
cinnamomea	FHC-1	Friday Harbor	11-12-60
sororia	FHS-1	Friday Harbor	11-6-60
sororia	FHS-2	Friday Harbor	11-6-60
sororia	FHS-3	Friday Harbor	11-12-60
hirsuta var. *maxima*	OHM-1	Olympia	10-27-60
mixtilis	TM-1	Tenino	10-30-60
xanthomelas	OXa-1	Olympia	11-12-60
nigrescens	FHN-1	Friday Harbor	11-12-60
nigrescens	FHN-2	Friday Harbor	11-6-60
nigrescens	ALN-1	Alaska[e]	7-10-61
albodisca	FHA-1	Friday Harbor	11-12-60
umbrina	UWU-1	U. of W. campus	12-1-60
1187	TPU-1	Tenino	10-30-60
4291	FHU-1	Friday Harbor	11-6-60
napipes	TN-1	Tenino	4-9-61
1540	FHNo-3	Friday Harbor	5-20-61
subexilis	FHSx-1	Friday Harbor	11-12-60

[a] Correct botanical designations of the named species are as follows: *I. picrosma* Stuntz, *I. terrifera* Kühn., *I. geophylla* (Fr.) Karst. var. *geophylla*, *I. lilacina* (Boud.) Kauff., *I. pudica* Kühn., *I. agglutinata* Peck, *I. subdestricta* Kauff., *I. griseolilacina* Lange, *I. lacera* (Fr.) Quél., *I. griseoscabrosa* (Peck) Earle, *I. pallidipes* Ellis and Everhart sensu Kauff., *I. cinnamomea* A. H. Smith, *I. sororia* Kauff., *I. hirsuta* var. *maxima* A. H. Smith, *I. mixtilis* (Britz.) Sacc. sensu Kühn., *I. xanthomelas* Boursier and Kühn. in Kühn., *I. nigrescens* Atk., *I. albodisca* Peck, *I. umbrina* Bres., *I. napipes* Lange, and *I. subexilis* (Peck) Sacc. [b] Deposited in the University of Washington mycological herbarium under this number (Stuntz No.). [c] Seattle. [d] University of Washington experimental forest, near Maltby. [e] Portage Glacier area, near Anchorage.

volumes of acetone (total 7 ml.) which were combined.

Quaternary reineckates contained in the acetone extract were converted to the respective chlorides (6) by adding 1.4 ml. of water, 5.0 ml. of a saturated solution of silver sulfate, and 0.5 ml. of a 20% aqueous solution of barium chloride. This mixture was cooled (12 hours), centrifuged, the supernatant liquid decanted and retained, and the precipitate washed with 5.0 ml. of cold water which was then added to the supernatant liquid. The combined solution was evaporated under reduced pressure at 50° in a flash evaporator and the residue dissolved in absolute ethanol (total volume 10 ml.). Purified extracts prepared in this manner were subjected to chromatographic examination.

Chromatographic Procedures.—A number of different solvent systems, including those employed by previous investigators (7, 8) for the separation of muscarine and other quaternary compounds, were examined to determine their suitability for use in a quantitative chromatographic procedure.

Satisfactory separation of muscarine and choline and discrete spots with minimum tailing were achieved when sheets of Whatman No. 1 filter paper, which had been previously dipped in MacIlvaine's buffer (9) at pH 4.5 and air dried, were formed with a wash liquid composed of *n*-butanol:methanol:water (10:3:2). Reference choline, acetylcholine, and muscarine exhibited average R_f values in this system of 0.09, 0.23, and 0.28, respectively. Since the alkaline extraction procedure employed resulted in the hydrolysis of any acetylcholine present in the mushroom tissues, this compound was not detected in the extracts and did not interfere with muscarine determination.

Quantitation of muscarine was carried out by spotting, in duplicate, suitable quantities (3–100 μl.) of the purified extract on a buffered chromatographic sheet. After formation, the sheet was air dried and sprayed uniformly with Thies and Reuther's reagent (10).

Preliminary investigations had revealed that the sensitivity of this reagent for muscarine varied somewhat according to the length of time following

its preparation from the stock solution but preceding its use. It was determined that maximum sensitivity was reached between 8 and 10 hours after mixing; consequently, the reagent was always prepared this length of time prior to use. Experiments with reference quantities of muscarine also revealed that following formation in the above system, detection of 5 mcg. of muscarine was equivocal, but 6 mcg. could be detected with certainty. This finding is in agreement with the report of Eugster (3).

After spraying with the reagent, the sheet was dried and subjected to careful visual inspection to determine the minimum amount of extract which formed a detectable orange-red spot. This observation was then verified by respotting, in duplicate, the same quantity on an additional sheet together with quantities of extract slightly smaller than, and slightly larger than, this amount. Actual quantities applied varied according to the concentration of muscarine in the extract. If the results of subsequent chromatograms verified the initial observations, the quantity of extract producing a just-detectable muscarine spot was considered equivalent

TABLE II.—CONCENTRATIONS OF MUSCARINE IN *Inocybe* SPECIES

Species of *Inocybe*	Collection No.	Mushroom Concentration of Purified Extract Gm./ml.	Extract Equivalent to 6 mcg. Muscarine,[a] μl.	Muscarine Content, %
picrosma	FHPi-1	1.35	50	<0.009[b]
		1.35	50	<0.009[b]
1790	UWP-1	1.17	50	<0.01[b]
		1.15	50	<0.01[b]
1838	FHG-1	1.93	12	0.03
		1.92	12	0.03
terrifera	OT-1	3.94	14	0.01
		5.29	10	0.01
geophylla	FHGG-1	0.47	8	0.16
		0.47	8	0.16
lilacina	FHL-1	0.26	6	0.38
		0.27	6	0.37
pudica	OP-1	0.90	4	0.17
		0.90	4	0.17
pudica	LPPu-1	0.69	7	0.12
		0.60	8	0.13
agglutinata	FHAg-1	0.38	5	0.31
		0.31	6	0.32
3691	FHH-1	0.51	15	0.08
		0.51	15	0.08
subdestricta	LFS-1	0.45	6	0.22
		0.45	6	0.22
2147	OSu-1	1.40	6	0.07
		1.35	6	0.07
2147	A2147-1	0.32	10	0.18
		0.35	10	0.17
3365	TSu-2	1.57	50	<0.009[b]
		1.50	50	<0.009[b]
griseolilacina	FHGr-1	0.33	11	0.17
		0.36	10	0.17
3399	FHNo-1	1.44	8	0.05
		1.45	8	0.05
3761	TO-1	1.82	3	0.11
		1.78	3	0.11
3761	OO-1	0.15	5	0.80
		0.16	5	0.75
2907	FHPo-1	0.25	8	0.30
		0.27	8	0.28
1774	FHNo-2	1.77	6	0.06
		1.80	6	0.06
3983	LPPg-1	0.95	5	0.13
		0.97	5	0.12
lacera	TL-1	1.44	5	0.08
		1.44	5	0.08
lacera	FHLa-1	0.40	18	0.08
		0.40	18	0.08
griseoscabrosa	FHGs-1	0.75	30	<0.02[b]
		0.95	30	<0.02[b]
pallidipes	LPP-1	0.48	8	0.16
		0.48	8	0.16
cinnamomea	FHC-1	1.43	14	0.03
		1.43	14	0.03
sororia	FHS-1	0.35	6	0.28
		0.36	6	0.28
sororia	FHS-2	0.86	4	0.17
		0.73	5	0.17
sororia	FHS-3	0.75	6	0.13
		0.75	6	0.13

TABLE II (*continued*)

Species of *Inocybe*	Collection No.	Mushroom Concentration of Purified Extract Gm./ml.	Extract Equivalent to 6 mcg. Muscarine,[a] µl.	Muscarine Content, %
hirsuta var. *maxima*	OHM-1	1.67	30	<0.01[b]
		1.60	30	<0.01[b]
mixtilis	TM-1	0.63	10	0.10
		0.66	9	0.10
xanthomelas	OXa-1	1.45	30	<0.01[b]
		2.15	30	<0.009[b]
nigrescens	FHN-1	1.93	30	<0.01[b]
		1.93	30	<0.01[b]
nigrescens	FHN-2	0.63	30	<0.03[b]
		0.62	30	<0.03[b]
nigrescens	ALN-1	0.71	100	<0.01[b]
		0.71	100	<0.01[b]
albodisca	FHA-1	2.10	30	<0.01[b]
		2.00	30	<0.01[b]
umbrina	UWU-1	0.73	3	0.27
		0.75	3	0.27
1187	TPU-1	0.97	6	0.10
		0.97	6	0.10
4291	FHU-1	1.43	4	0.11
napipes	TN-1	0.14	6	0.71
		0.14	6	0.71
1540	FHNo-3	0.63	4	0.24
		0.65	4	0.23
sibexilis	FHSx-1	1.07	30	<0.01[b]
		1.20	30	<0.02[b]

[a] If muscarine was not detected, this was the largest volume spotted. [b] Muscarine not detected. The maximum quantity which could be present but remain undetected has been derived by calculation.

to 6 mcg. of muscarine. From these data, the concentrations of muscarine in the dried carpophores were calculated. If the maximum quantity of purified extract spotted did not produce a detectable muscarine spot, the maximum concentration which could be present and remain undetected was calculated. These data are reported in Table II.

In order to determine the relative efficiency and accuracy of the entire procedure (extraction, purification, and chromatographic estimation), 7.4 mg. of muscarine was added to a sample (4.41 Gm.) of *I. nigrescens*, a species in which muscarine could not be detected. This control sample was treated as previously described, and the amount of muscarine present in the purified extract was found to be equivalent to 7.0 mg., approximating a 95% recovery of the added compound.

Pharmacological estimations of the muscarine content of certain of the identical collections employed in this investigation have been carried out by Malone, *et al.* (11). With the exception of the assays of *I. napipes*, which apparently contains other potent muscarine-like agents or potentiators, the results of the biological and chromatographic assays were in general agreement.

DISCUSSION

Comparison of the muscarine contents of the species investigated in this study with proposed infrageneric divisions of *Inocybe* based on classical morphological and histological criteria (2) reveals little in the way of a definite relationship between muscarine content and taxonomic position. Certain generalizations may be made, such as the probable occurrence of appreciable muscarine concentrations in species centered about the stirps geophylla Heim of the section Viscosae Heim (*I. geophylla* var. *geophylla*, *I. lilacina*, *I. pudica*, *I. agglutinata*) or the various stirpes of the section Gibbosporae Heim (*I. umbrina*, *I. napipes*, Nos. 1187, 4291, 1540). There are, however, cases in which no correlation is possible such as the easily detectable occurrence of muscarine in *I. mixtilis* but its apparent absence in the closely related *I. xanthomelas* and *I. nigrescens*.

While muscarine has been shown to be of widespread occurrence in the genus *Inocybe*, its occurrence within the various subdivisions of the genus is apparently not entirely predictable on taxonomic grounds. This does not lessen the significance of the compound as a taxonomic criterion in those species in which it occurs, but it does reduce its relative importance as a genus characteristic. Some evidence obtained in this investigation by analysis of different collections of the same species indicates that the capacity of *Inocybe* species to accumulate muscarine may be influenced by certain hereditary (infraspecific races) or environmental factors.

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Effect of Sulfate Content of Several Anionic Polymers on *in Vitro* Activity of Pepsin

By L. J. RAVIN, J. G. BALDINUS, and M. L. MAZUR

Carrageenan was degraded with acid to give materials with sulfate contents varying from 5–30%. Antipeptic activities of these materials correlated closely with the sulfate content. Other sulfated polymers such as polyvinyl sulfonate, sulfated rice starch, amylose sulfate, heparin, etc., gave a similar correlation. All these polymers precipitated protein in the antipeptic test. Compounds such as sodium lauryl sulfate and *m*-xylene sulfonate possessed significant antipeptic activity but did not precipitate protein, and no correlation between sulfate content and antipeptic activity was found. The precipitation of protein by carrageenan depends upon the pH, becoming negligible at a pH above 4.5.

THAT SULFATED polysaccharides possess antienzyme activity has been established by a number of workers. Levey and Sheinfeld (1) found that the proteolytic action of pepsin on casein is inhibited by chondroitin sulfate, heparin, and sodium polyanhydromannuronic acid sulfate. Fellig and Wiley (2) demonstrated that heparin and various synthetic anionic polymers are inhibitors of pancreatic ribonuclease. More recently, Anderson and Watt (3, 4) and Houck, Bhayana, and Lee (5) reported that the incidence of ulcers in stress rats and in cortisone- and histamine-treated dogs and rats can be reduced by the oral administration of carrageenan, a sulfated polymer. Anderson (6) states that the degree of peptic inhibition parallels the sulfate content of the polymer. This paper deals with the effect of the sulfate content of several synthetic and naturally occurring anionic polymers on the *in vitro* activity of pepsin with emphasis placed on carrageenan and its degraded forms.

Carrageenan is a heterogenous, water-soluble polysaccharide obtained from marine algae such as *Chondrus crispus*. The two main components are kappa- and lambda-carrageenan, which can be separated by selective precipitation with potassium ions. These components differ in molecular size, viscosity, optical rotation, sugar units, and sulfate content (7–16).

Carrageenan is sensitive to acid. The glycosidic bonds undergo hydrolysis under relatively mild conditions, whereas the sulfate groups require more drastic conditions for their removal. This principle has been utilized by Evans Medical Limited to make C-16, a form of carrageenan with lower viscosity than the starting material yet with essentially the same sulfate content (17). Because of its low viscosity, C-16 is easier to administer orally (6). In the present study C-16 has been subjected to further acid hydrolysis; we will refer to the resulting materials with varying sulfate content as degraded carrageenan. The remaining sulfated polymers used in this study were obtained through commercial sources or through the generosity of several individuals. Amylose sulfate was prepared by the method of Roubal, Placer, and Vokac (18).

Received December 1, 1961, from the Smith Kline & French Laboratories, Philadelphia, Pa.
Accepted for publication February 16, 1962.
The authors are indebted to the Microanalytical Chemistry Group at Smith Kline & French Laboratories for the sulfate analyses reported in this paper; to Marine Colloids, Inc., Rockland, Me. for the supply of lambda- and kappa-carrageenan; to P. Bernfeld of the Bio-Research Institute, Cambridge, Mass. for the sulfated rice starch; and to J. Fellig of Union Carbide Chemical Co., South Charleston, W. Va., for the supply of polyvinyl sulfonate.

EXPERIMENTAL

Materials.—C-16, carrageenan, Evans Medical Ltd., Speke, Liverpool 24, England; lambda-carrageenan; kappa-carrageenan; Sea Kem, type 8, Marine Colloids, Inc., Rockland, Me.; pectin N.F.; sulfated rice starch; polyvinyl sulfonate; amylose sulfate; heparin sodium, 100 units/mg., Nutritional Biochemicals Corp., Cleveland, Ohio; sulfated polyvinyl alcohol, Chemicals Procurement Co., New York 36, N. Y.; polyvinyl alcohol; sodium alginate; carboxymethylcellulose; sodium lauryl sulfate, U.S.P.; sodium heptadecyl sulfate; sodium *m*-xylene sulfonate; sodium 1,2-ethylene disulfonate dihydrate; alginic acid sulfate, Naurylande SARL, Venetta, France; pepsin, 1–10,000, Nutritional Biochemicals Corp., Cleveland, Ohio; hemoglobin, lyophilized, salt free, Mann Research Laboratories, New York, N. Y.; albumen (bovine) fraction V, Nutritional Biochemicals Corp., Cleveland, Ohio; blood plasma, normal, human, irradiated, dried, Hyland Laboratories, Los Angeles, Calif.

Degradation of Carrageenan.—C-16 (100 Gm.) was suspended in a 10% (v/v) solution of concentrated hydrochloric acid in acetone. The suspension was shaken periodically to facilitate hydrolysis. The resulting material was collected on filter paper, washed with anhydrous acetone, dissolved in a minimum quantity of water, and the solution adjusted to pH 7.0 with 40% (w/v) sodium hydroxide. After treatment with charcoal, the solution was filtered and the clear filtrate was added to anhydrous acetone, with constant stirring, until precipitation was complete. The precipitate was washed with anhydrous acetone, dried overnight at 50° and analyzed for antipeptic activity and sulfate content. By varying the time of hydrolysis, carrageenans with different sulfate contents were obtained.

Determination of Antipeptic Activity.—Hunt's (19) method was modified as follows: each run required a control, a carrageenan standard arbitrarily designated as having a 100% antipeptic activity, the unknown, and the appropriate blanks. The control consisted of 1 ml. pepsin, 1 ml. human plasma, and 1 ml. acidified water; the standard had 1 ml. pepsin, 1 ml. plasma, and 1 ml. carrageenan standard; the unknown, 1 ml. pepsin, 1 ml. plasma, and 1 ml. test substance. Concentrations were: pepsin, 0.25 mg./ml.; human plasma, 5%; test substance and standard, 0.5%; pH of these solutions was 1.6 adjusted with hydrochloric acid only.

The mixtures were incubated at 37°; after exactly 1 hour, 10 ml. trichloracetic acid (10% aq.) was added; 15 minutes later, the mixtures were filtered. To 1 ml. filtrate were added: 10 ml. aq. sodium hydroxide (0.4 *N*) and 3 ml. dilute (1 part reagent and 2 parts water) Folin-Ciocalteu reagent. After 15–20 minutes, the absorbances (*A*) were read on a Klett-Summerson colorimeter (No. 66 filter). Results were calculated using the following equation

$$\frac{A \text{ control} - A \text{ test substance}}{A \text{ control} - A \text{ standard carrageenan}} \times 100 = \% \text{ antipeptic activity}$$

When hemoglobin was used to check the method, the antipeptic values were similar to those obtained with human plasma.

Determination of Interaction with Protein.—One-half per cent solutions of the sulfated polymers were adjusted to pH 1.6, 3.0, 4.0, 4.5, and 5.0, respectively, with hydrochloric acid. Then, 4 ml. of each solution was mixed with 1 ml. of 5% solutions of blood plasma; albumen, bovine, fraction V; and hemoglobin; similarly adjusted to the same pH values with hydrochloric acid. The resulting mixtures were kept at 37° for 1 hour, then they were filtered and the tyrosine in the filtrate was measured colorimetrically, as described above. This method does not differentiate between unreacted protein and a soluble protein-sulfated polymer complex.

Determination of Sulfate.—Samples were burned in a Schöniger flask which contained 0.01*N* sodium hydroxide as an absorbent solution. After combustion was complete, the flask contents were shaken and allowed to stand for 1 hour to insure complete absorption of the oxides of sulfur. After treatment with fuming nitric acid, the contents were evaporated to dryness and the residue dissolved in water and neutralized to a phenolphthalein end point. The resulting sulfate was titrated with barium chloride solution (0.02 *N*) using tetrahydroxyquinone as indicator (20–22).

RESULTS AND DISCUSSION

***Interaction between Protein and Carrageenan* (C-16) at Various pH's.**—It is thought that carrageenan exerts its antipeptic activity *in vitro* by precipitating the protein, and *in vivo* by forming a protective coating on the stomach walls (6). Carrageenan does not precipitate pepsin, and recently Piper and Fenton (23), on the basis of paper electrophoresis studies, concluded that there is no interaction at all between pepsin and carrageenan. Carrageenan precipitates protein by virtue of its many negative charges which can interact with positively charged proteins to form insoluble complex salts. The ability of carrageenan to precipitate several proteins has been determined at various pH's; these data are summarized in Table I. It is apparent that protein precipitation depends upon the pH and that the amount of precipitation decreases as the pH rises. At pH 4.5 and higher, little or no protein is precipitated. The reaction between carrageenan and protein is completely reversible; a protein precipitated with carrageenan can be brought back into solution simply by raising the pH. These facts suggest that antacids may be contraindicated when carrageenan is administered.

TABLE I.—EFFECT OF PH ON THE INTERACTION BETWEEN C-16 AND SEVERAL PROTEIN SUBSTRATES

	Interaction, %		
pH	Blood Plasma	Albumen (Bovine) Fraction V	Hemoglobin
1.6	100	100	100
3.0	99	83	79
3.5	...	75	82
4.0	88	8	39
4.5	47	...	...
5.0	0	0	0

Sulfate Content *vs.* Antipeptic Activity.—C-16 was hydrolyzed with acid to decrease the sulfate content and was then assayed for antipeptic activity. Table II lists the sulfate content and antipeptic activity of several degraded samples. These data are also plotted in Fig. 1. It is clear that the antipeptic activity correlates closely with sulfate content. At the lower sulfate contents, however, the antipeptic activity tends to level off.

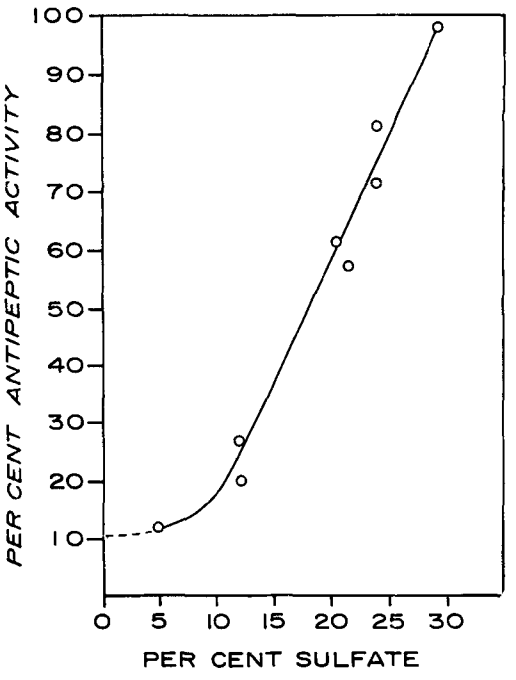


Fig. 1.—Relationship between *in vitro* antipeptic activity and sulfate content for degraded carrageenan.

TABLE II.—RELATIONSHIP BETWEEN SULFATE CONCENTRATION AND ANTIPEPTIC ACTIVITY FOR DEGRADED CARRAGEENAN

Hydrolysis Time, hr.	Sulfate, %	Antipeptic Activity, %
0.0	29.0	98.5
0.5	23.7	81.0
1.0	23.7	71.0
3.0	21.4	57.0
6.0	20.3	61.0
24.0	12.0	27.0
48.0	12.2	20.0
96.0	4.9	12.0

Sulfate Content of Various Natural and Synthetic Polymers *vs.* Antipeptic Activity.—The fact that carrageenan's antipeptic activity parallels its sulfate content prompted us to test other sulfate and sulfonated polymers with varying degrees of sulfation. Table III lists the polymers tested, their antipeptic activity, and their sulfate content. In all cases, the antipeptic activity is that compared with a standard sample of C-16. Figure 2 is a plot of these data. Again, the antipeptic activity correlates with the sulfate content; *i.e.*, the greater the sulfate content, the greater the antipeptic activity.

Since all the polymers precipitated protein in the antipeptic test we can assume that the mechanism of pepsin inhibition is similar to that of carrageenan. In summary, then, for those polymers that precipitate protein, the antipeptic activity is completely dependent upon the sulfate content. Other chemical or physical properties of these polymers may come into play, but these effects are not apparent by the methods used in this study.

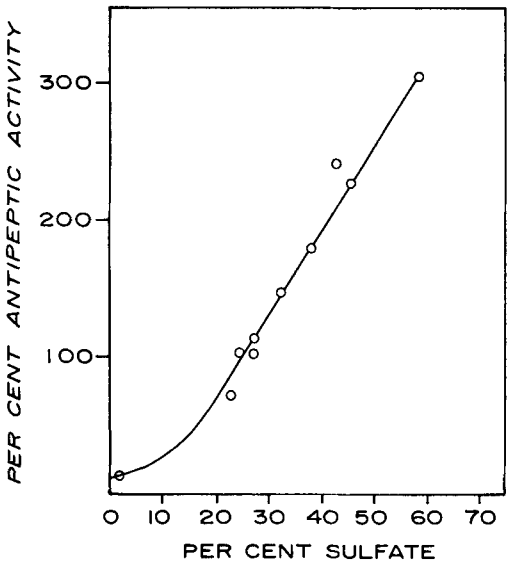


Fig. 2.—Relationship between *in vitro* antipeptic activity and sulfate content for several anionic polymers.

Comparison of Antipeptic Activity of Lambda- and Kappa-Carrageenan Mixtures.—The effect of varying ratios of lambda- to kappa-carrageenan on the *in vitro* activity of pepsin is shown in Fig. 3 which shows that the higher the concentration of lambda fraction the greater the antipeptic activity. Kappa- and lambda-carrageenan differ greatly in viscosity, yet their antipeptic activity correlates with their sulfate content (Table III). *In vivo*, it is possible that the viscosity of carrageenan may have a bearing on its antipeptic activity; *in vitro*, however, it is not the viscosity but the sulfate content that determines its antipeptic effectiveness.

TABLE III.—RELATIONSHIP BETWEEN SULFATE CONTENT AND ANTIPEPTIC ACTIVITY FOR SEVERAL NATURALLY OCCURRING AND SYNTHETIC POLYMERS

Polymer	Antipeptic activity, %	Sulfate, %
Polyvinyl sulfonate	310	59.0
Sulfated polyvinyl alcohol	247	42.5
Sulfated rice starch	230	45.3
Amylose sulfate	182	38.0
Lambda-carrageenan	150	32.2
Kappa-carrageenan	110	24.0
Heparin	103	27.0
Carrageenan, C-16	114	27.1
Sodium alginate	13	0
Carboxymethylcellulose	0	0
Polyvinyl alcohol	15	0
Pectin N.F.	12.5	2.07
Sea Kem, type 8	73	23.0
Alginic acid sulfate	40	15.8
Carrageenan, standard	100	29.0

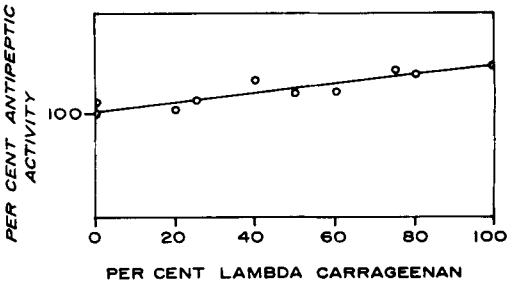


Fig. 3.—Effect of various ratios of lambda- to kappa-carrageenan on the *in vitro* activity of pepsin. Concentration is expressed as per cent lambda-carrageenan.

Compounds That Did Not Precipitate Protein.—During our screening program we found several nonpolymeric compounds with very high antipeptic activities that did not correlate with the sulfate content (Table IV). Since these compounds did not precipitate protein in the antipeptic test it can be assumed that they interact with protein or pepsin to form soluble complexes. In these interactions, factors such as the conformation of the compounds, the size of the micelles in solution, etc., probably predominate so that the role played by the sulfate content becomes insignificant.

TABLE IV.—ANTIPEPTIC ACTIVITY AND SULFATE CONTENT OF COMPOUNDS THAT DID NOT PRECIPITATE PROTEIN

	Sulfate, %	Antipeptic Activity, %
Sodium lauryl sulfate	31.0	408
Sodium heptadecyl sulfate	41.36	240
Sodium *m*-xylene sulfonate	12.84	93
Sodium 1,2-ethylene disulfonate dihydrate	71.50	29

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Synthesis of Some New Derivatives of N^4-*p*-Cyanobenzoyl Sulfanilamide

By LAWRENCE J. FISCHER and BERNARD ECANOW

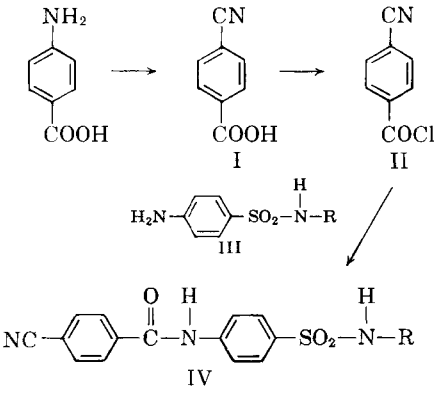
Six new derivatives of N^4-*p*-cyanobenzoyl sulfanilamide were prepared: N^4-*p*-cyanobenzoyl sulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrimidinylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-(4-methyl-2-pyrimidinyl)sulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-thiazolylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyridylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrazylsulfanilamide. Infrared spectra of the new compounds were determined. Common absorption bands of the compounds occurred at 2240–2210 cm.$^{-1}$, 1160–1130 cm.$^{-1}$, 1325–1310 cm.$^{-1}$, and 1580–1560 cm.$^{-1}$.

N^4-SUBSTITUTED sulfanilamides have shown important pharmacological actions. N-Sulfanilylbenzamide, which is similar to the derivatives prepared here, has been shown to be effective against the bacillary dysentery organism (1) and against pneumococcus in mice (2).

Some N - alkyl - *p* - cyanobenzamides have been prepared and shown to possess local anesthetic and antimicrobial properties (3). The amidines and imidazolines prepared from these compounds have also shown significant activity (4).

The synthesis of some new sulfonamides containing the *p*-cyanobenzoyl and sulfanilyl moieties was successfully completed.

The synthesis of the N^4-*p*-cyanobenzoyl sulfanilamide derivatives was initiated with the preparation of *p*-cyanobenzoic acid I, which was prepared according to the procedure of Miller and Gisvold (5). *p*-Cyanobenzoyl chloride II was prepared by refluxing the acid with thionyl chloride and recrystallizing from petroleum ether.



Equimolar quantities of *p*-cyanobenzoyl chloride and the finely powdered sulfanilamide derivative III were refluxed in *p*-dioxane for 2 hours. Mechanical stirring was required if the reactants were not completely soluble in dioxane. The solvent was removed and the N^4-*p*-cyanobenzoyl sulfanilamide derivative IV was treated with dilute-hydrochloric acid to remove any unreacted material. Recrystallization was from a mixture of solvents.

The solubility characteristics of the new derivatives (see Table I) were generally predictable.

Received November 4, 1961, from the College of Pharmacy, University of Illinois, Chicago Professional Colleges, Chicago.
Accepted for publication November 29, 1961.
Abstracted from a thesis submitted by Lawrence J. Fischer to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science, 1961.

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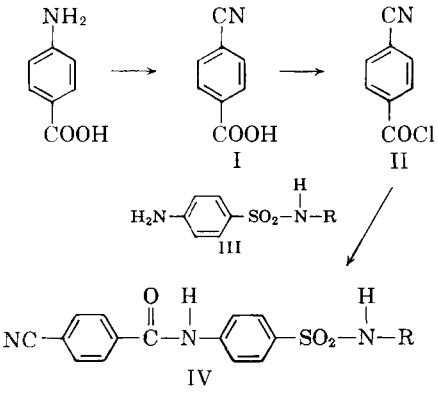
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The synthesis of some new sulfonamides containing the *p*-cyanobenzoyl and sulfanilyl moieties was successfully completed.

The synthesis of the N^4-*p*-cyanobenzoyl sulfanilamide derivatives was initiated with the preparation of *p*-cyanobenzoic acid I, which was prepared according to the procedure of Miller and Gisvold (5). *p*-Cyanobenzoyl chloride II was prepared by refluxing the acid with thionyl chloride and recrystallizing from petroleum ether.

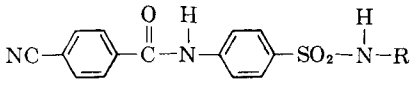


Equimolar quantities of *p*-cyanobenzoyl chloride and the finely powdered sulfanilamide derivative III were refluxed in *p*-dioxane for 2 hours. Mechanical stirring was required if the reactants were not completely soluble in dioxane. The solvent was removed and the N^4-*p*-cyanobenzoyl sulfanilamide derivative IV was treated with dilute-hydrochloric acid to remove any unreacted material. Recrystallization was from a mixture of solvents.

The solubility characteristics of the new derivatives (see Table I) were generally predictable.

Received November 4, 1961, from the College of Pharmacy, University of Illinois, Chicago Professional Colleges, Chicago.
Accepted for publication November 29, 1961.
Abstracted from a thesis submitted by Lawrence J. Fischer to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science, 1961.

TABLE I.—N^4-*p*-CYANOBENZOYLSULFANILAMIDE DERIVATIVES



R	Formula	Yield, %	M.p., ° C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
—H	$C_{14}H_{11}N_3O_3S$	62.2	277–280	55.81	55.79	3.68	3.70	...	...
	$C_{18}H_{13}N_5O_3S$	70.1	317–318 (decompn.)	57.00	56.96	3.45	3.61	18.44	18.51
	$C_{19}H_{15}N_5O_3S$	78.4	295–297 (decompn.)	58.01	57.89	3.84	4.09	...	...
	$C_{17}H_{12}N_4O_3S_2$	75.2	286–288 (decompn.)	53.10	53.22	3.15	3.29	...	...
	$C_{19}H_{14}N_4O_3S$	83.5	286–288 (decompn.)	60.35	60.48	3.73	3.75	14.81	14.93
	$C_{18}H_{13}N_5O_3S$	82.6	290–291	56.99	57.07	3.45	3.42	18.46	18.36

They were found to be insoluble in the common laboratory solvents such as water, alcohol, ether, and chloroform. The products were soluble to sparingly soluble in *p*-dioxane, and very soluble in dimethylacetamide. Salt formation occurred readily in sodium hydroxide solution, the sodium salts being soluble in water.

EXPERIMENTAL

Infrared spectra were obtained using a Beckman IR-4 infrared recording spectrophotometer. All of the compounds prepared exhibited the characteristic cyano band in the range 2240–2210 cm.$^{-1}$. The spectra of the *p*-cyanobenzoyl sulfanilamide derivatives contained absorption bands common to each member of the series. Such bands occurred at 1160–1130 cm.$^{-1}$, 1325–1310 cm.$^{-1}$, and 1580–1560 cm.$^{-1}$

Preparation of *p*-Cyanobenzoic Acid and *p*-Cyanobenzoyl Chloride.—This was done according to the procedure of Miller and Gisvold (*5*).

Preparation of N^4-*p*-Cyanobenzoyl sulfanilamide.—Four grams of powdered sulfanilamide U.S.P. (0.0242 mole) and 4.1 Gm. (0.0242 mole) of *p*-cyanobenzoyl chloride were placed in a 500-ml., round-bottomed flask fitted with a reflux condenser and mechanical stirrer. The solvent, 270 ml. of *p*-dioxane, was added and the suspension refluxed, while stirring slowly, for 1.5 hours.

The reaction mixture was allowed to cool to room temperature. The solid product formed during the reaction was filtered with suction. The crude product was treated with two 75-ml. portions of 10% hydrochloric acid by mixing in a mortar, filtered with suction, and washed with distilled water after each treatment. The product was dried under vacuum and weighed 4.5 Gm.

Recrystallization was from a dimethylacetamide-distilled water mixture (1:1). The recrystallized product was placed in an oven at 200° for 1.5 hours to remove any adhering solvent.

N^4-*p*-Cyanobenzoyl-N^1-2-pyrimidinyl sulfanilamide.—Sulfadiazine U.S.P., 10.0 Gm. (0.08 mole), and 13.2 Gm. (0.08 mole) of *p*-cyanobenzoyl chloride were placed in a 500-ml., round-bottomed flask, fitted with a reflux condenser and a mechanical stirrer. A 200-ml. quantity of *p*-dioxane was added and the mixture refluxed, while stirring slowly, for 1.5 hours. The reaction mixture was allowed to stand overnight and the product filtered with suction. The product, a white powder, was allowed to dry at room temperature. It was then treated with 150 ml. of 10% hydrochloric acid by mixing in a mortar, filtered with suction, and washed with generous amounts of distilled water. The product was recrystallized from dimethylacetamide.

N^4-*p*-Cyanobenzoyl-N^1-(4-methyl-2-pyrimidinyl)-sulfanilamide.—To a 500-ml., round-bottomed flask equipped with a reflux condenser, 3.44 Gm. (0.013 mole) of sulfamerazine U.S.P. powder and 2.16 Gm. (0.013 mole) of *p*-cyanobenzoyl chloride were added. A 125-ml. quantity of *p*-dioxane was added and a clear, brown, solution resulted. The solution was refluxed for 2 hours and cooled to room temperature. The solvent was removed on a flash evaporator and the residue recrystallized from a dioxane-water mixture.

N^4-*p*-Cyanobenzoyl-N^1-2-thiazolylsulfanilamide.—Sulfathiazole U.S.P. powder, 1.4 Gm. (0.0055 mole), and 0.9 Gm. (0.0055 mole) of *p*-cyanobenzoyl chloride were added to a 250-ml., round-bottomed flask equipped with a reflux condenser. The solvent, 70 ml. of *p*-dioxane, was added and a clear solution was obtained. The solution was refluxed for 1.75 hours and allowed to cool to room temperature.

The solvent was removed on a flash evaporator and the residue treated with 75 ml. of 4% hydrochloric acid by mixing in a mortar. The product was recrystallized by dissolving it in approximately 3 ml. of dimethylacetamide, adding 25 ml. of 95% ethanol, and adding distilled water to the hot solution until the cloudiness just disappeared upon heating. The

light tan crystals were placed in an oven at 165° for 1 hour to remove any adhering solvent.

N^4-*p*-Cyanobenzoyl-N^1-2-pyridylsulfanilamide.—To a 250-ml., round-bottomed flask, equipped with a reflux condenser and a mechanical stirrer, was added 1.52 Gm. (0.00606 mole) of sulfapyridine U.S.P. powder and 1.0 Gm. (0.00606 mole) of *p*-cyanobenzoyl chloride. Seventy milliliters of *p*-dioxane was added and the mixture refluxed, while slowly stirring, for 2 hours.

The reaction mixture was allowed to cool to room temperature and the solvent removed on a flash evaporator. The residue was treated successively with two 100-ml. portions of 4% hydrochloric acid by mixing in a mortar, filtering, and washing with distilled water.

The product was recrystallized by dissolving in 5 ml. of hot dimethylacetamide, then adding 20 ml. of dioxane, and finally adding distilled water until the cloudiness produced just disappeared upon heating. The pale yellow crystals were dried in an oven at 130° to remove any adhering solvent.

N^4-*p*-Cyanobenzoyl-N^1-2-pyrazylsulfanilamide.—Three grams (0.0121 mole) of sulfapyrazine powder and 2.0 Gm. (0.0121 mole) of *p*-cyanobenzoyl chloride were added to a 500-ml., round-bottomed flask, equipped with a mechanical stirrer and a reflux condenser. The solvent, 140 ml. of *p*-dioxane, was added and the mixture refluxed, while stirring, for 2 hours.

The dioxane was removed on a flash evaporator and the residue mixed with 100 ml. of 10% hydrochloric acid in a mortar. The product was filtered with suction, washed with distilled water, and the acid treatment repeated.

The product was recrystallized from a solution consisting of one part dimethylacetamide, four parts dioxane, and four parts of distilled water. The pale yellow crystals were placed in an oven at 140–150° for one-half hour to remove any adhering solvent.

SUMMARY

1. Six new derivatives of N^4-*p*-cyanobenzoyl sulfanilamide were prepared: N^4-*p*-cyanobenzoylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrimidinylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-(4-methyl-2-pyrimidinyl)sulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyridylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-pyrazylsulfanilamide, N^4-*p*-cyanobenzoyl-N^1-2-thiazolyl-sulfanilamide.

2. The infrared spectra of the new compounds were determined. Common absorption bands of the compounds occurred at 2240–2210 cm.$^{-1}$, 1160–1130 cm.$^{-1}$, 1325–1310 cm.$^{-1}$, and 1580–1560 cm.$^{-1}$.

3. The results of the pharmacologic investigation of the new compounds will be reported in a forthcoming paper.

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Cyclized Substituted Thioureas II

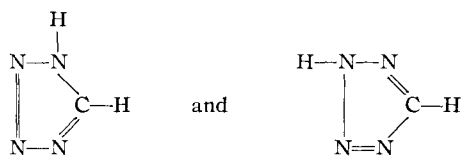
Preparation of Some 1-Substituted 1,2,3,4-Tetrazole-5-thiones

By RONALD E. ORTH† and JAMES W. JONES

Some 1-alkyl- and 1-aryl-1,2,3,4-tetrazole-5-thiones are synthesized by preparing methyl-(N-alkyl- and N-aryl)-dithiocarbamates by dropwise addition of carbon disulfide to a cold solution of the amine in aqueous sodium hydroxide, followed by slowly esterifying, separating, and purifying the ester. Sodium azide is refluxed with the various esters forming, in several cases, the substituted tetrazole-5-thione on purification.

PREVIOUSLY it has been demonstrated that thiourea has great antithyroidal activity. It appears that the introduction of this moiety into a ring system causes a general decrease in toxicity. However, conjugation of a second ring decreases the physiological activity with respect to the single ring systems. In addition, it has been found that at least one of the thiourea nitrogen must contain hydrogen. This leads investigators to hypothesize that tautomerism within the H—N—C=S portion of the molecule is paramount to good suppression of iodine-uptake.

The synthesis of tetrazole (1) led to much investigation regarding the various substitution products which could be obtained. The parent compound melts at 155° and exists in two isomeric forms



Received November 13, 1961, from the College of Pharmacy, State University of Iowa, Iowa City.
Accepted for publication November 26, 1961.
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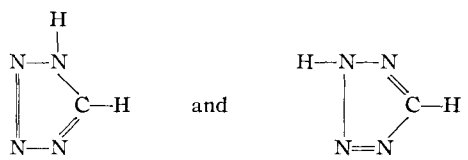
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By RONALD E. ORTH† and JAMES W. JONES

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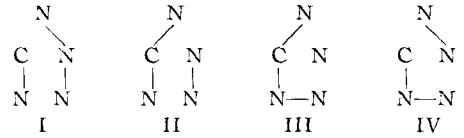
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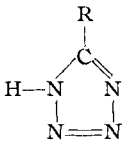
Theoretically, there are four general methods for the preparation of a tetrazole. The following examples show the nuclei needed for all possible combinations



I II III IV

Specifically, tetrazole and its derivatives have been prepared by method I by condensation of an azoimide with prussic acid and its derivatives and by the reaction of the imidochlorides and similar compounds with sodium azide. The reaction of nitrous acid with hydrazadines or amidines, followed by reduction, follows the method III scheme. The condensation of phenylazoimide with aldehyde phenylhydrazones, using sodium alcoholate in ethanol, and the reaction of diazohydrazides and mono- and diacylhydrazines with diazonium salts in the presence of alkali are method IV syntheses. Method II, as yet, has not been used for the preparation of the tetrazole nucleus. The oxidation of suitable tetrazolium compounds is another approach.

Some of the tetrazole derivatives which have been synthesized are illustrated by the general formula



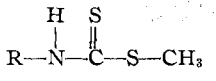
5-R-1,2,3,4, 1-H-tetrazoles where R may be phenyl, tolyl, furyl, anisyl, bromo, amino, or mercapto

With the knowledge that pentylenetetrazole U.S.P. is used in medicine for its central nervous system stimulation in certain mental disorders, particularly in schizophrenia and depressive psychoses (2), it is felt that the tetrazole nucleus is one which should be investigated more extensively in conjunction with pharmaceuticals. Pentylenetetrazole has also been used as an antidote in poisoning by depressant drugs, however it appears to be inferior to picrotoxin (3).

A number of methyl-(N-alkyl- and N-aryldithiocarbamates) have previously been prepared (4). This paper deals in part with the preparation of several new esters, the attempt to purify and characterize these, and a few prepared in earlier investigations.

The esters of N-alkyl- and N-aryldithiocarbamate prepared were used to synthesize some 1-aryl-and 1-alkyl-1,2,3,4-tetrazole-5-thiones not previously reported.

TABLE I.—METHYL ESTERS OF N-ALKYL- AND N-ARYLDITHIOCARBAMIC ACID PREPARED



	Nitrogen, % Calcd.	Found	Yield, %[a]	B.P., °C./mm.
H[b]	13.3	12.9	60	...
Methyl[b]	11.6	12.0	40	153–156/20
Ethyl	10.4	10.1	35	115/8
n-Propyl	9.39	9.22	81	124/8
Isopropyl	9.39	9.13	49	76/5
n-Butyl	8.59	8.40	63	...
Cyclohexyl[b]	7.41	7.78	68	89/5
Benzyl[b]	7.10	6.89	45	...
Phenyl[b]	7.65	7.48	34	97[c]

[a] Based on 0.5 mole of amine. [b] Previously prepared. [c] Melting point.

EXPERIMENTAL

Methyl Esters of N-Aryl- and N-Alkyldithiocarbamic Acid.—In a 1-L. round-bottom, three-necked flask, surrounded by an ice bath and fitted with a mechanical stirrer, a reflux condenser, thermometer, and a 250-ml. dropping funnel, were placed a cold solution of 72 Gm. (1.8 mole) of sodium hydroxide in 160 ml. of water and 1.8 moles of primary amine. To this mixture, cooled to 0–10°, 137 Gm. (1.8 mole) of carbon disulfide was added dropwise with continuous stirring. The reaction was exothermic and the mixture turned red in color. When the reaction subsided, 1.8 moles of methyl iodide was added dropwise over a period of 1 hour and, as the reaction proceeded, the color disappeared. The procedure used to separate the esters from the reaction mixtures varied slightly with each ester as follows:

Methyl-N-ethyldithiocarbamate was obtained by repeatedly extracting the reaction mixture with small portions of carbon disulfide or carbon tetrachloride, evaporating the solvent, and washing with water. The thick, yellow oil was fractionally distilled under reduced pressure.

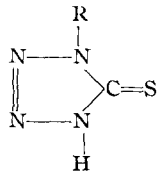
Methyl-N-n-propyldithiocarbamate was obtained by extracting the reaction mixture with many small portions of diethylether, drying over calcium chloride, filtering, and evaporating the solvent. A good yield of thick, yellow oil was fractionally distilled under reduced pressure.

Methyl-N-isopropyldithiocarbamate was prepared by refluxing the mixture for 24 hours after the addition of the methyl iodide. Separation and purification was carried out in the same manner as described for methyl N-*n*-propyldithiocarbamate.

Methyl-N-n-butyldithiocarbamate also required refluxing for 24 hours after the addition of methyl iodide. The ester was isolated from the reaction mixture and distilled, as described under methyl-N-*n*-propyldithiocarbamate. The analyses and boiling points of the methyl esters of N-alkyl- and N-aryldithiocarbamic acid are given in Table I.

1-Ethyl-1,2,3,4-tetrazole-5-thione was prepared by dissolving 24.3 Gm. (0.18 mole) of methyl-N-ethyldithiocarbamate and 8.1 Gm. (0.18 mole) of sodium azide in 100 ml. of 95% ethanol, then diluting to 130 ml. with water to solubilize the inorganic azide. Warming aided in effecting solution at this point. The solution was refluxed over a water bath at 60°

TABLE II.—1-ALKYL- AND 1-ARYL-1,2,3,4-TETRAZOLE-5-THIONES PREPARED



R	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Yield, %	M.P., °C.
Methyl	...	...	...	...	48.3	49.1	7.00	124–126
Ethyl	27.6	27.4	4.61	4.57	43.0	42.5	24.00	240–245
n-Propyl	33.2	33.0	5.54	5.62	38.4	37.7	22.00	74– 76
Cyclohexyl	...	...	...	...	30.5	30.5	...	102–103
Benzyl	...	...	...	...	29.2	28.4	43.00	145–146
Phenyl	...	...	...	...	31.5	31.9	48.00	152

for several hours, then cooled in an ice bath and acidified. The cold mixture was filtered and the clear filtrate was reduced in volume by evaporation on a steam bath. At the first sign of crystallization, the concentrated filtrate was slowly cooled allowing fine, white, needle-like crystals to precipitate. The crystals were washed with a small portion of water and, upon drying, were taken up in absolute alcohol and precipitated by the addition of absolute ether. The pure crystals melted at 240–245°.

1-n-Propyl-1,2,3,4-tetrazole-5-thione was prepared by dissolving 7.5 Gm. (0.05 mole) of methyl-N-*n*-propyldithiocarbamate and 3.3 Gm. (0.05 mole) of sodium azide in 30 ml. of 95% ethanol. Enough water was added to bring the azide into solution. The reaction mixture was refluxed for 2 hours then cooled in ice, acidified, and filtered. The filtrate was concentrated to small volume. Upon cooling the tetrazole was separated as long, white needles. After purifying in the same manner as used for the ethyl analog, crystals melted at 76°.

The analyses and melting points of 1-alkyl- and 1-aryl-1,2,3,4-tetrazole-5-thiones are given in Table II.

DISCUSSION

A search of the literature revealed that many of the methyl esters of N-alkyl- and N-aryldithiocarbamic acid had previously been prepared as intermediates, but few physical constants were tabulated. It was also found in the preparation of the esters that as the alkyl chain of the primary amine was lengthened, the reactivity with carbon disulfide in aqueous sodium hydroxide decreased slightly and the reactivity with the methylating agent decreased markedly. The methylating agents used, in the order of their reactivity, were methyl iodide, methyl sulfate, and methyl *p*-toluenesulfonate. It was found that primary aliphatic amines with alkyl chains longer than four carbons did not yield appreciable amounts of methyl esters.

In an attempt to find a more general method for the preparation of 1-alkyl- and 1-aryl-1,2,3,4-tetrazole-5-thiones, phenylisothiocyanate was reacted with sodium azide giving near quantitative yields of the phenyl derivative of the tetrazole-5-thiones (5). 1-Allyl-1,2,3,4-tetrazole-5-thione was prepared from allyl isothiocyanate and sodium azide in low yields (6).

It was found that the methyl esters of N-isopropyl-, N-*n*-butyl-, and N-isobutyldithiocarbamic acid did not react under the given conditions to form the corresponding tetrazoles. It became apparent that an increase in chain length and branching decreased the reactivity of the isothiocyanate with sodium azide.

SUMMARY

1. Eight N-substituted methyl esters of dithiocarbamic acid were prepared. Of these, the ethyl, *n*-propyl, isopropyl, *n*-butyl-, and cyclohexyl- had not been reported.

2. Six 1-alkyl- and 1-aryl-1,2,3,4-tetrazole-5-thiones were prepared from the corresponding dithiocarbamate esters. Of these, the ethyl and *n*-propyl had not been reported.

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Specificity of the Relationship between Rate of Dissolution and Disintegration Time of Compressed Tablets

By L. C. SCHROETER, J. E. TINGSTAD, E. L. KNOECHEL, and J. G. WAGNER

Dissolution rates and disintegration times of 76 lots of tablets were determined employing the U.S.P. disintegration apparatus without plastic disks. The time for 50% of the drug to dissolve ($t_{50\%}$) or the amount of drug in solution at a specific time was used as criteria of the rate of dissolution. Disintegration times were also obtained with plastic disks. The drugs included a steroid, a sulfonamide, an antidiabetic agent, and an aspirin-phenacetin-caffeine combination. In some cases there was a quantitative relationship between $t_{50\%}$ and one or both of the disintegration times. In other cases there was none. Where a quantitative relationship existed, the slopes of the lines relating the variables varied widely and depended upon the drug(s) involved, and in one case upon the presence of a pharmaceutical adjuvant. With two of the series of tablets, one could not differentiate between lots on the basis of their disintegration times determined with plastic disks. However, in one of these series disintegration times were determined without plastic disks, and in both the $t_{50\%}$ values clearly identified lot-to-lot variation. Questions are raised regarding the use of plastic disks in the official disintegration test and the validity of applying uniform disintegration standards to large groups of drugs in tablets. An explanation for the correlation between *in vivo* availability of certain drugs administered as tablets to human subjects and their disintegration times is presented.

SIXTY YEARS ago Hance (1) defined solubility as applied to compressed tablets to indicate the "power to disintegrate rather than the power to form solutions." This is regrettable terminology. Sperandio, *et al.* (2), pointed out that disintegration of a tablet does not necessarily mean that the drug has dissolved; the tablet is merely broken up into smaller particles when it disintegrates. Nair and Bhatia (3) showed that changing the screen in the U.S.P. disintegration apparatus from a No. 10 mesh to a No. 20 mesh screen could markedly alter observed disintegration times. Parrott, *et al.* (4), stated that "while the disintegration time of a tablet does influence the rate of drug release to the body, a most important aspect is the rate of release from the primary drug particle since solution of the drug is essential in order for absorption to take place."

Nelson (5) and Levy (6) pointed out that rapid disintegration of tablets has been equated by some workers with rapid availability of the drug for absorption. In his excellent review of the history of committee and official action on tablet disintegration tests, Vliet (7) quoted from the April 1949 Contact Committee meeting minutes as follows: "in setting up a list of recommended disintegration times, it was felt that the primary purpose of such a limit is to insure to the user of the tablet that ultimate availability of the medication and in such reasonable time as the nature of the medication might warrant." With a series of sugar-coated tablets containing riboflavin and another series containing sodium *p*-aminosalicylate, Chapman, *et al.* (8, 9), showed that the availability of these drugs for absorption was predictable from the disintegration time of the tablets determined by a modified U.S.P. XIV disintegration test. The modifications involved employing a 3-L. instead of a 1-L. beaker, using rubber disks which exerted a rubbing action on the tablets, and using a simulated gastric juice, pH 1.6, for the first half-hour followed by simulated intestinal juice, pH 8.0. Subsequently, Endicott and Kirchmeyer (10) presented a literature study and new experimental data as evidence that tablets requiring considerably more than 1 hour to disintegrate *in vitro* by the U.S.P. XV method may still be satisfactory drug sources in the human body. With a series of five commercial aspirin tablets, Levy (6) showed that their disintegration times had no relation whatsoever to rate of absorption or to biological availability of the aspirin in human subjects. However, the amount of aspirin in solution after 10 minutes, which was measured in a standardized *in vitro* test and used as a criterion of the rate of dissolution, did correlate linearly with the amount of salicylate excreted during the first hour following administration of the tablets.

Perhaps the reason for this discrepancy in

Received January 10, 1962, from the Department of Product Research and Development, The Upjohn Co., Kalamazoo, Mich.

Accepted for publication January 29, 1962.

The authors acknowledge the excellent technical assistance of Jane Patterson and G. R. Munting.

The authors express their appreciation to the Control Division of The Upjohn Co. for performing the disintegration tests with plastic disks on the tablets containing the antidiabetic agent.

results lies in the specificity of any correlation between either rate or extent of availability *in vivo* and either rate of dissolution or disintegration time determined by a particular *in vitro* method. Assuming that the rate and/or the extent of availability of a certain drug administered in tablets to human subjects will correlate with the *in vitro* rates of dissolution, then the *in vivo* data will also correlate with results of some disintegration test providing the specific dissolution test correlates with the specific disintegration test.

This hypothesis requires experimental proof. In this communication evidence is presented that the nature of the drug, the tablet formulation, and the method of manufacture will determine whether a correlation exists between any two of the variables: rate of dissolution, disintegration time determined without disks, and disintegration time determined with disks. If such a correlation exists, these same factors will also influence the quantitative relationship between the variables. In future communications data will be presented which also support the concept of specificity in correlations between *in vivo* availability and either rate of dissolution or disintegration time determined by particular methods.

Suggestions have been made (6, 11, 12) that the U.S.P. tablet disintegration test be replaced by a dissolution test, and opinions were recently requested (13) on this subject. Before such a change is made, or even seriously considered, it appears to be desirable to test the above hypothesis. If each drug incorporated into a given series of compressed tablets is an individual problem then the attempt to establish uniform standards for large numbers of drugs in many types of compressed tablets is spurious.

EXPERIMENTAL

Tablets.—All tablets were from experimental lots prepared in the process of developing satisfactory formulas for possible commercial use. Series of 12 lots of compressed tablets containing an anti-inflammatory steroid, seven lots of compressed tablets containing a sulfonamide, seven lots of compressed tablets containing an antidiabetic agent, and 16 lots of compressed tablets and one lot of granulation containing a combination of aspirin, phenacetin, and caffeine were studied. Details pertaining to the exact formulation and their methods of manufacture would add nothing to the test results and the conclusions drawn and, hence, are omitted. In one series, the tablets containing the sulfonamide, the presence of one ingredient, sodium chloride, influenced the nature of the statistical correlations presented. This was taken into consideration in interpreting the results.

Dissolution and Disintegration Tests Determined without Plastic Disks.—The disintegration time, without plastic disks, was always determined during the same test in which the fluid was being sampled to obtain rate of dissolution.[1] Hence, all original dissolution data refer to test conditions employing the U.S.P. XV tablet disintegration apparatus. The buffers used in these tests are shown in Table I. When performing such tests, care must be taken that the dissolution medium does not become saturated with the drug before all the dose is released from the tablet or tablets being tested. It is most desirable to arrange the dissolution test so that when all the drug has been released from the sample, the fluid is only 25%, or less, saturated with respect to the drug. If this is the case, then the plots of amount or per cent of drug in solution against time will not be influenced appreciably by this factor, but rather reflect the nature of the tablet itself. The approximate per cent saturations of the buffers with the drugs at the end of the tests are shown in Table I.

In the case of the series of tablets of the anti-inflammatory steroid, one test was performed per lot of tablets, but each test involved five tablets. For two of the lots a repeat test was run. For all other tablet lots only one tablet was tested at a time, but two separate tests were performed on each lot. Tablets were chosen randomly from the lots for the dissolution-disintegration tests with the exception of the tablets containing aspirin-phenacetin-caffeine. In the latter case, tablets of the lot were chosen which had individual weights and thicknesses as closely as possible to the average weight and thickness of the particular lot.

Without stopping the agitation of the U.S.P. tablet disintegration apparatus, aliquots of the dissolution medium were withdrawn at different times through pipets which were wound with glass wool at their tips. When the filtered aliquots had cooled to room temperature they were either transferred directly to 1-cm. silica cells or repipetted and appropriately diluted with the same buffer used as dissolution medium. The filtered aliquots or their dilutions were scanned in a Cary recording spectrophotometer, model 11, over the wavelength region 210 to 400 mμ. The absorbances at the wavelength of maximum absorption of the unknown and standard were used to calculate the concentration of drug. In calculating the amount of drug released at a given time, corrections were applied for the amounts of drug in previously withdrawn aliquots. In the case of the aspirin-phenacetin-caffeine combination, the percentage of drug released at a given time, t, was calculated from the expression

$$\% \text{ drug released} = A_t/A_{t_\infty} \times 100 \quad \text{(Eq. 1)}$$

[1] Apparently, Klein (14), in 1932, was the first investigator to determine the rate of dissolution of a compressed tablet. A year later, Elliott (15), using the same apparatus, which he called a Solvometer, published plots of amount of drug dissolved against time following tests on compressed tablets of five different drugs; he attempted "to show the influence exerted upon the rate of solution by two important variables —temperature and surface in contact with the liquid." Elliott's tablets apparently had essentially constant surface areas since he found the rates of solution were constant until about nine-tenths of the tablets were dissolved. Prior to Klein and Elliott, Noyes and Whitney (16), in 1897, derived their law governing rate of dissolution by studying dissolution from constant surface cylinders of pure benzoic acid in water. Subsequent to Klein and Elliott's investigations, Parrott, Wurster, and Higuchi (17) studied dissolution from spherical tablets of pure benzoic acid. Nelson (18) studied dissolution from cylindrical tablets of pure compounds. To our knowledge, Chapman, Crisafio, and Campbell (9) were the first investigators to determine rates of dissolution at the same time that the distintegration time was being determined; they obtained curved plots similar to some of those illustrated in this paper.

TABLE I.—BUFFERS, NUMBER OF TABLETS PER TEST, NUMBER OF TESTS PER LOT OF TABLETS, AND APPROXIMATE PER CENT SATURATION OF BUFFER WITH DRUG IN THE DISSOLUTION AND DISINTEGRATION TESTS

	Rate of Dissolution and Disintegration Tests without Plastic Disks				Disintegration Tests with Plastic Disks	
Type of Drug in Tablets	Buffer Used[a]	No. of Tablets per 750 ml. of Buffer Held in 1-L. Beaker	No. of Tests per Lot	Approximate % Saturation of Buffer with Drug When Tablet(s) all Dissolved	Fluid Used	No. of Tablets (Vol. Fluid)
Anti-inflammatory steroid	HCl-NaCl, pH 1.3 (μ = 0.1)	5	1	22.7	U.S.P. XVI, pH 1.2[b]	6 (1 L.)
Sulfonamide	Phosphate, pH 7.2	1	2	11.5	...	...
Antidiabetic agent	Tris(hydroxymethyl)-aminomethane-HCl pH 7.2	1	2	12 to 15	Water	6 (1 L.)
Phenacetin-aspirin-caffeine combination	HCl-NaCl, pH 1.3 (μ = 0.1)	1	2	*ca.* 6% aspirin *ca.* 8% phenacetin *ca.* 1% caffeine	HCl-NaCl, pH 1.3 (μ = 0.1)	6 (750 ml.)

[a] Buffers and dissolution media were maintained at 37.5 ± 2° during the tests. [b] Simulated gastric fluid T.S. U.S.P. XVI.

where A_t and A_{t_∞} were the absorbances at 230 mμ at time t and at a time when all the aspirin, phenacetin, and caffeine in the tablet had dissolved, respectively. It was shown that when aspirin, phenacetin, and caffeine were mixed in the same ratio as they existed in the tablet, a single absorption maximum at 230 mμ was obtained, whereas the individual components gave absorption maxima at 227, 243, and 272 mμ, respectively.

The other ingredients in the tablets introduced less than 1% error; this is well within the error of the ultraviolet spectrophotometric method. Greater errors were introduced by evaporation and mechanical loss of the dissolution media during prolonged tests. This error amounted to as much as about 10% in the apparent total amount of the antidiabetic agent released from a tablet. However, these tests were carried out for a period of 4 hours or more. Loss of fluid during the first hour was measured. The volumes of fluid lost during the first half-hour and one hour under the conditions employed were about 30 and 40 ml., respectively, or 4 and 5%, respectively, of the initial 750 ml. volume. Hence, the maximum error introduced in any value obtained at a half-hour was 4% and at one hour was 5% of the amount or per cent of drug released at these times. Tests performed on all tablets, other than the antidiabetic agent, were essentially complete after 1 hour. The $t_{50\%}$ values used in the correlations were in the ranges 1 to 14 minutes for the anti-inflammatory steroid and the sulfonamide tablets, and 1 to 38 minutes for the aspirin-phenacetin-caffeine tablets.

Disintegration Times Determined with Plastic Disks.—Disintegration times were determined for the anti-inflammatory steroid, antidiabetic agent, and phenacetin-aspirin-caffeine series of tablets using the U.S.P. XVI tablet disintegration apparatus and the official plastic disks. The fluids used in the individual tests are shown in Table I.

Lack of correlation between rate of dissolution (determined without plastic disks) and disintegration time (determined with plastic disks) in the case of the anti-inflammatory steroid cannot be attributed to the small differences between the buffers used in the two tests. The solubility and rate of dissolution of this particular anti-inflammatory steroid are independent of the pH and such small changes in ionic strength.

Data from the Literature.—Some correlation coefficients were calculated from the data of Chapman, Crisafio, and Campbell (8, 9).

RESULTS AND DISCUSSION

Tablets of Anti-inflammatory Steroid.—Plots of per cent drug in solution against time in minutes are shown in Fig. 1. From these plots the time required for 50 and 100% of the anti-inflammatory steroid contained in the tablets to dissolve was estimated. These times are shown in Table II. The range of disintegration times and the average disintegration time obtained during the dissolution tests are also shown in Table II. The time required for 50% of the drug to dissolve ($t_{50\%}$) is plotted

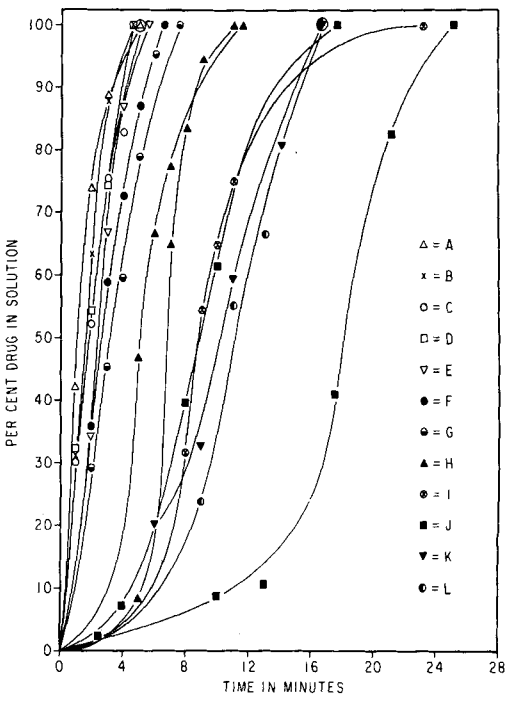


Fig. 1.—Plots of per cent drug in solution against time in minutes for tablets containing the anti-inflammatory steroid.

TABLE II.—ESTIMATED TIMES REQUIRED FOR 50 AND 100% OF THE DRUG TO DISSOLVE, AVERAGE DISINTEGRATION TIMES OBSERVED DURING DISSOLUTION TESTS, AND DISINTEGRATION TIMES BY U.S.P. PROCEDURE FOR TABLETS CONTAINING THE ANTI-INFLAMMATORY STEROID

Lot	Estimated Time Required for 50% of Drug to Dissolve,[a] min.	Estimated Time Required for 100% of Drug to Dissolve,[a] min.	Disintegration Time[a] Determined without Plastic Disks during Dissolution Test, min. Range	Average	Av. Disintegration Time of Six Tablets when Plastic Disks Were Used in U.S.P. Test, min.
A	1	4	2 to 4	3	4
B	1.5	4.5	2 to 4	3.6	5
C	1.5	5	3 to 4	3.6	2
D	2	4.5	3 to 4	3.6	3
E	2	5.5	4 to 5	4.6	4
F	2.5	6.5	3 to 6	4.4	5
G	3	7.5	5 to 7	6	5.5
H[b]	5	11.5	5 to 11	7	
H[b]	7	11	5 only	5	6.5
I	9	23	8 to 23	12	7.5
J[b]	9	17.5	8 to 17.5	12	6
J[b]	18	25	16 to 25	21	
K	10	16.5	5 to 16	11	5.5
L	11	16.5	9 to 16	13	6.5

[a] Based on five randomly selected tablets. [b] Repetitive tests with same lot were performed on separate days.

against the average disintegration time determined during the dissolution tests without plastic disks in Fig. 2. There is a highly significant linear correlation ($P < 0.001$) between the two quantities. The slope of the least squares lines is approximately unity, but there is a significant negative intercept. Since $t_{50\%}$ is a reasonably good criterion of the rate of dissolution, it may be concluded that for this series of 12 lots of tablets of the anti-inflammatory steroid that the rate of dissolution of these tablets is linearly correlated with the average disintegration time obtained under the test conditions. However, use of plastic disks, as in the official U.S.P. disintegration test, masks significant differences between the lots of these tablets. There is no correlation between disintegration times obtained for this series of tablets with the U.S.P. procedure and any criterion of the rate of dissolution such as $t_{50\%}$. These results are different than those reported at the November 1954 Contact Committee meeting (7). At that time it was reported that there was very little difference in time with and without disks, using tablets that disintegrated readily.

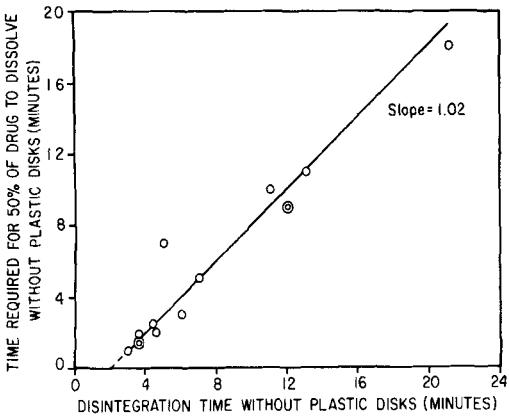


Fig. 2.—Correlation of time required for 50% of drug to dissolve with disintegration time for tablets containing an anti-inflammatory steroid. Each point represents the time required for 50% of the drug contained in five tablets to dissolve and the average disintegration time of the same five tablets observed during the dissolution test.

Tablets Containing the Sulfonamide.—Typical plots of per cent drug in solution against time in minutes are shown in Figs. 3 and 4. Figure 4 is a plot of part of the same data as shown in Fig. 3, but the time scale has been expanded to show more clearly differences between lots in the first 15 minutes of the dissolution tests. From these and similar plots the average time required for 25, 50, 75, and 90% of the sulfonamide contained in the tablets to dissolve was estimated. These times are shown in Table III together with the average of the disintegration times of the two tablets of each lot observed during the dissolution tests. The $t_{50\%}$ value for each individual tablet is plotted against the corresponding disintegration time observed

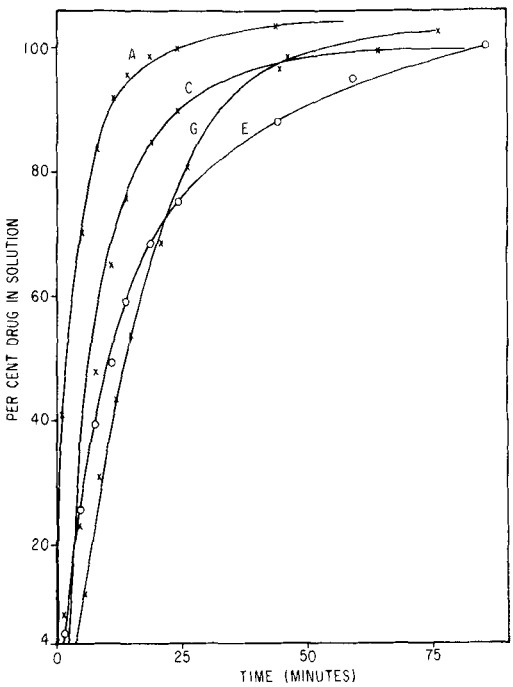


Fig. 3.—Plots of per cent drug in solution against time in minutes for some of the tablets containing the sulfonamide.

during the dissolution test in Fig. 5. The points in Fig. 5 fall into two different populations. In each population there is a highly significant linear correlation between $t_{50\%}$ and disintegration time. The line with slope of 1.82 includes the two tablets of lots A, B, D, and E; all tablets in this series contained sodium chloride as an ingredient. The line with slope of 1.10 includes the two tablets of each of lots C, F, and G; all tablets in this series contained no sodium chloride. These results indicate that correlations between rate of dissolution (using $t_{50\%}$ as a criterion of rate) and disintegration time are highly specific and may be dependent not only upon the type of drug but also upon the nature of pharmaceutical adjuvants in the tablets.

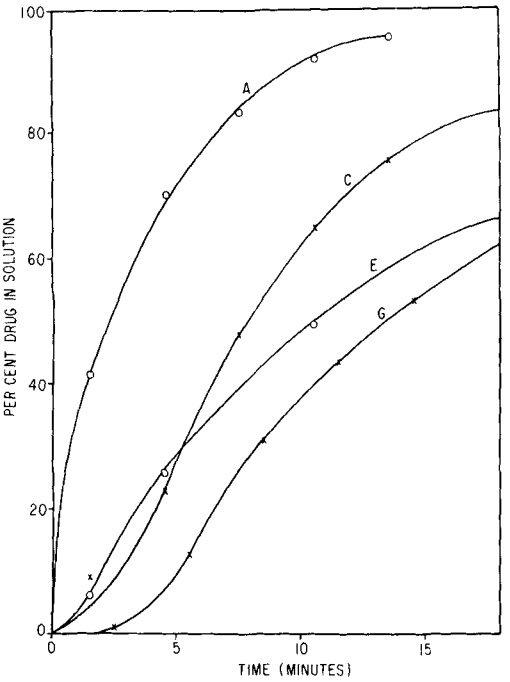


Fig. 4.—Plots of per cent drug in solution against time in minutes for some of the sufonamide tablets; data for first 15 min. only are plotted.

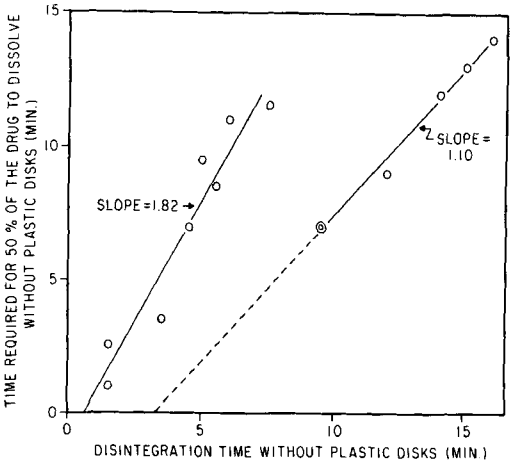


Fig. 5.—Correlation of time required for 50% of drug to dissolve with disintegration time for tablets containing a sulfonamide. Each point represents a single tablet. The line with slope of 1.82 resulted from testing two tablets of each of lots A, B, D, and E; all tablets in this series contained sodium chloride as an ingredient. The line with slope of 1.10 resulted from testing two tablets of each of lots C, F, and G; all tablets in this series contained no sodium chloride.

Tablets Containing the Antidiabetic Agent.—Plots of apparent milligrams of drug in solution against time in hours for all the tablets tested are shown in Figs. 6 and 7. Two tablets of each of the seven lots were tested. The data for the more rapidly dissolving tablets of each lot are plotted in Fig. 6; the data for the more slowly dissolving tablet of each lot are plotted in Fig. 7. The data were not converted to percentages in this case since insufficient assays were performed in some cases to obtain the 100% values. Errors were introduced due to loss in volume of dissolution medium by evaporation during the prolonged dissolution tests. For this reason the word "apparent" has been used.

TABLE III.—AVERAGE TIME REQUIRED FOR THE DIFFERENT PERCENTAGES OF DRUG TO DISSOLVE AND AVERAGE DISINTEGRATION TIME WITHOUT DISKS FOR DIFFERENT LOTS OF SULFONAMIDE TABLETS[a]

Lot	Av. Time Required for the Indicated Percentage of Drug to Dissolve, min. 25%	50%	75%	90%	Av. Disintegration Time without Disks, min.
A	1.0	2.0	5.0	9.5	1.5
B	1.5	5.0	13.5	30.5	4.0
C	5.0	8.0	13.0	24.0	9.5
D	4.5	10.0	19.5	34.5	6.0
E	4.5	10.5	23.5	48.0	6.0
F	6.5	11.0	19.0	30.0	13.5
G	7.5	13.5	23.0	32.5	15.0

[a] Time estimated to nearest 0.05 minute.

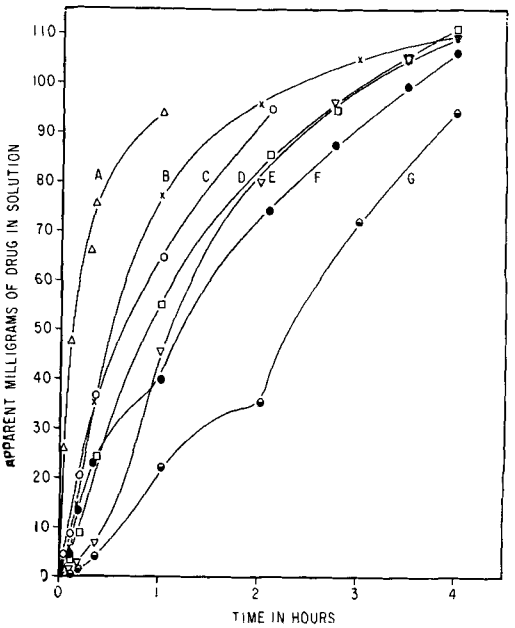


Fig. 6.—Plots of apparent milligrams of drug in solution against time in hours for the more rapidly dissolving tablet of each pair of tablets of each lot of tablets of antidiabetic agent tested.

TABLE IV.—AVERAGE POTENCIES, DISINTEGRATION TIMES WITH AND WITHOUT PLASTIC DISKS, AND ESTIMATED APPARENT MILLIGRAMS OF DRUG IN SOLUTION AT VARIOUS TIMES DURING THE DISSOLUTION TESTS FOR TABLETS OF THE ANTIDIABETIC AGENT

Lot	Average Potency, mg. drug/tab.	Av. Disintegration Time when Disk Used, min.	Dissolution Tests on Individual Tablets: Tablet No.	Disintegration Time without Disks, min.	Estimated Apparent mg. of Drug in Solution at Indicated Time, hr.: 0.25 hr.	0.50 hr.	1 hr.	2 hr.	3 hr.	4 hr.
A	97.0	0.5	1	0.5	76	81	94	..	...	...
			2	1	30	41	70	78	...	...
B	98.2	0.7	1	90	27	51	77	96	104	109
			2	90	23	36	58	83	97	106
C	89.6	2	1	50	16	30	57	89	...	...
			2	50	26	45	71	92	...	...
D	101.5	4	1	167	14	27	48	77	93	102
			2	130	14	34	55	82	98	111
E	101.8	3	1	140	5	12	46	80	100	109
			2	170	3	7	25	70	91	102
F	100.3	1.5	1	20	21	29	40	73	91	106
			2	21	15	22	29	42	52	60
G	102.9	3	1	490	3	7	16	33	54	72
			2	360	3	7	22	35	72	93

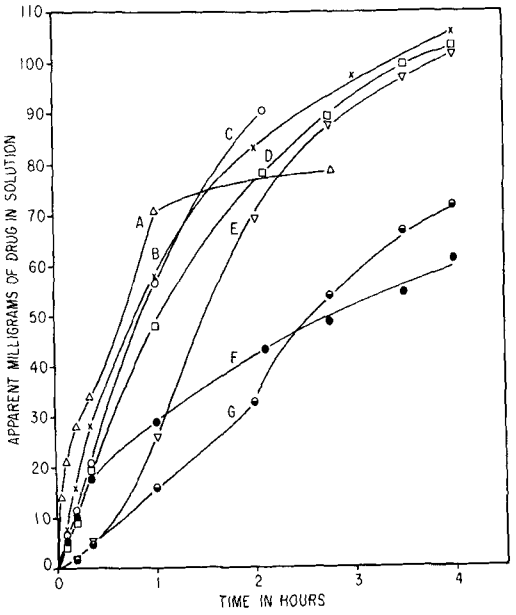


Fig. 7.—Plots of apparent milligrams of drug in solution against time in hours for the more slowly dissolving tablet of each pair of tablets of each lot of tablets of antidiabetic agent tested.

However, such loss in volume of fluid and subsequent error in the amount of drug in solution was not appreciable during the first hour of the tests. From the plots shown in Figs. 5 and 6, the apparent milligrams of drug in solution at 0.25, 0.50, 1, 2, 3, and 4 hours were estimated; these values are shown in Table IV. The disintegration times of the individual tablets observed during the dissolution tests and the average disintegration time obtained using the U.S.P. procedure are also shown in Table III.

Similar to the $t_{50\%}$ value, the apparent milligrams of drug in solution at a given time should be a useful criterion of the rate of dissolution. However, no suitable correlation of the amount of drug in solution at a given time and the disintegration time of the tablet observed during the dissolution test was obtained with this series of tablets. Although there was some tendency for the more rapidly dissolving tablets to disintegrate and to pass through the screen of the apparatus sooner than the more slowly dissolving tablets, there were exceptions and no suitable quantitative correlation could be found. The U.S.P. XVI disintegration test gave results which masked the differences between lots of tablets. This can be seen by comparing the U.S.P. disintegration times shown in Table IV with the dissolution data shown in the same table or with the plots shown in Figs. 6 and 7.

Tablets Containing Aspirin-Phenacetin-Caffeine.—Typical plots of per cent drug in solution against

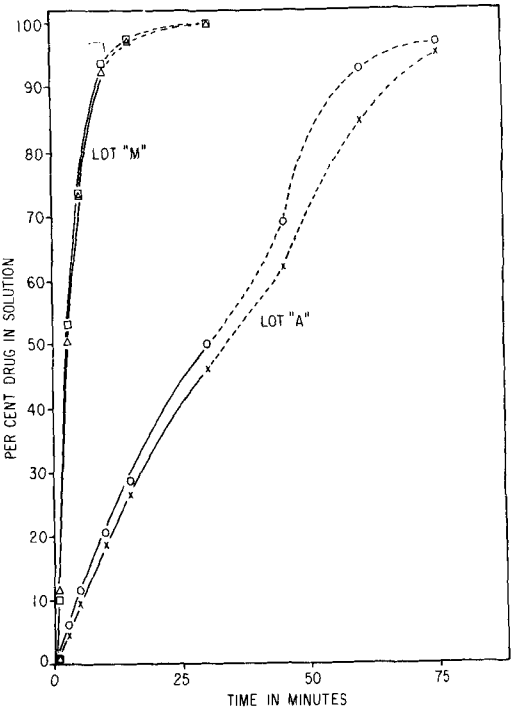


Fig. 8.—Plots of per cent drug in solution against time in minutes for the most rapidly dissolving lot (M) and the most slowly dissolving lot (A) of the tablets containing aspirin-phenacetin-caffeine.

time in minutes are shown in Fig. 8. Of the 16 lots of tablets and 1 lot of granulation tested, tablet lot M was the most rapidly dissolving and tablet lot; A was the most slowly dissolving lot (see Table V). The initial portion of the dissolution curves for this series was found to give excellent pseudo first-order plots. For example, the points joined by solid lines in Fig. 8 are replotted in Fig. 9. The slopes of the linear segments were obtained by the method of "least squares." From these slopes, or pseudo first-order rate constants, the half-lives were calculated. Since there was usually a brief lag time before the tablet started to release drug to solution at a pseudo first-order rate, the half-life does not correspond exactly with the time required for 50% of the drug to dissolve. The latter values were also estimated from the type of plots shown in Fig. 8. The relationship between the time required for 50% of the drug to dissolve and the half-life calculated from the pseudo first-order rate constant is shown in Fig. 10. The slope of the "least squares" line through the origin in Fig. 9 is 1.07; this indicates that, in general, the $t_{50\%}$ value is slightly greater than the half-life.

The $t_{50\%}$ values for this series are plotted against the disintegration times of the tablets observed during the dissolution tests in Fig. 11. The slope of the "least squares" line is 0.56 which is distinctly different from the slope of 1.02 found for the tablets of the anti-inflammatory steroids and the slopes of 1.10 and 1.82 found for the two series of tablets containing the sulfonamide.

The average disintegration time determined on six tablets using plastic disks is plotted against the average disintegration time of two tablets determined without plastic disks in Fig. 12. The slope of the "least squares" line through the origin is 0.67; this indicates that, in general, the effect of the plastic disks, with this series of tablets, was to decrease the disintegration time to about two-thirds of the time obtained without disks. Because of the significant linear correlations shown in Figs. 10, 11, and 12, it is obvious there are also significant linear correlations for this series between either $t_{50\%}$ or half-life and average disintegration time determined with plastic disks.

Tablets Containing Riboflavin.—The data of Chapman, Crisafio, and Campbell (8) were used. A plot of the average disintegration time determined with rubber disks against the average disintegration time determined without rubber disks is shown in Fig. 13. The values for lot 17 were omitted in estimating the regression. Although the slope (0.62) of the "least squares" line in Fig. 13 is similar to that (0.66) found for the aspirin-phenacetin-caffeine combination in our studies, the intercepts of two plots are significantly different. The slopes of the lines forced through the origin are 0.72 for the

TABLE V.—DISINTEGRATION TIMES WITH AND WITHOUT PLASTIC DISKS, TIME REQUIRED FOR DIFFERENT PERCENTAGES OF DRUG TO DISSOLVE, AND HALF-LIVES CALCULATED FROM THE DISSOLUTION DATA FOR TABLETS CONTAINING ASPIRIN, PHENACETIN, AND CAFFEINE

Lot	Av. (and Range) of Disintegration Times when, Disks Used, Min.	Dissolution Tests on Individual Tablets: Tablet No.	Disintegration Time without Disks, min.	Time for the Indicated Percentage to Dissolve, min.: 50%	70%	80%	90%	Half-Life, min.
A	35.3	1	59	34	51	57	64	32.9
	(28 to 41)	2	53	30	46	51	57	29.5
B	33.8	1	55	38	47	52	58	31.6
	(20 to 40)	2	53	32	47	52	58	28.4
C	32.5	1	45	29	42	48	57	23.3
	(28 to 36)	2	59	34	51	58	61	32.5
D	33.2	1	47	27	37	43	52	26.9
	(30 to 35)	2	46	25	37	44	54	24.5
E	27.2	1	44	28	38	42	49	24.1
	(23 to 32)	2	41	22	34	39	47	19.7
F	27.8	1	38	18	..	..	..	18.8
	(25 to 32)	2	38	18	31	37	42	17.5
		3	37	23	34	39	44	22.3
G	24.8	1	35	21	33	37	42	18.7
	(22 to 30)	2	31	19	29	34	40	18.2
H	23.8	1	31	23	34	39	44	21.3
	(20 to 26)	2	31	22	33	38	44	21.3
I	20.7	1	27	15	23	27	33	15.0
	(16 to 28)	2	27	14	21	25	29	12.2
J	13.7	1	21	14	20	24	28	13.9
	(11 to 18)	2	17	10	15	18	23	8.38
K	4.7	1	16	12	16	19	22	13.1
	(3 to 8)	2	14	8	12	14	17	7.25
L	3.3	1	6	6	8	9	13	3.34
	(2 to 4)	2	4	4	6	8	11	2.81
M	2.5	1	2	3	5	6	9	2.53
	(2 to 4)	2	2	3	5	6	9	2.32
N	1.0	1	1	2.5	4	6	8.5	2.87
	(1)	2	1	2.5	5	6	9	2.95
O	1.2	1	1	2	4	6	8.5	2.51
	(1 to 2)	2	1	2	4	6	8.5	2.39
P	1.0	1	1	3	5.5	7.5	10	2.13
	(1)	2	1	2	4	5.5	9	2.45
Granulation	0	1	0	2	4	6	8.5	2.81
		2	0	1.25	3.5	6	8.5	3.02

riboflavin tablet data and 0.67 for the aspirin-phenacetin-caffeine data. This is remarkable correspondence in the effect of the disks in these two series when one takes into consideration the marked differences in the other experimental conditions of the disintegration tests in the two series. It should be mentioned, however, that in the riboflavin tablets series, rubber disks were used whereas in the aspirin-phenacetin-caffeine series, plastic disks were used. In a series of seven sugar-coated tablets of sodium

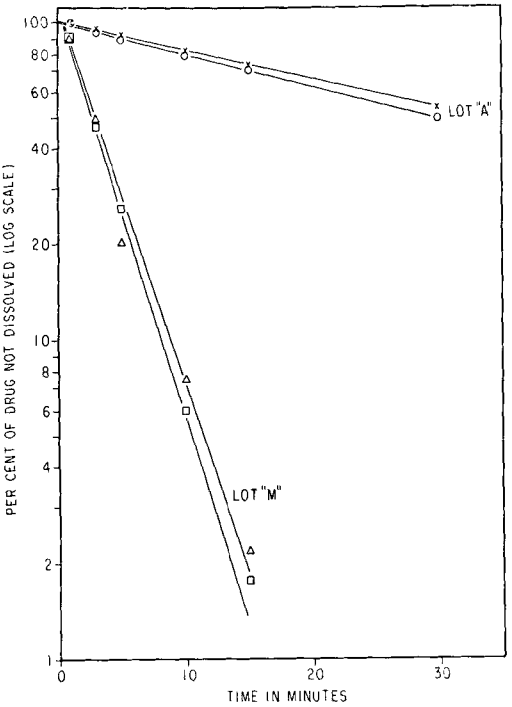


Fig. 9.—Initial pseudo first-order plots for dissolution of tablets containing aspirin-phenacetin-caffeine.

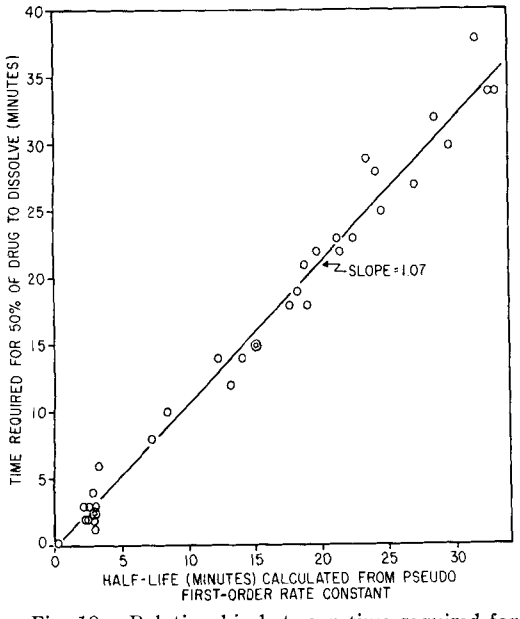


Fig. 10.—Relationship between time required for 50% of the drug to dissolve (estimated from dissolution rate plot such as shown in Fig. 8) and the half-life calculated from the initial pseudo first-order rate constant for tablets containing aspirin-phenacetin-caffeine.

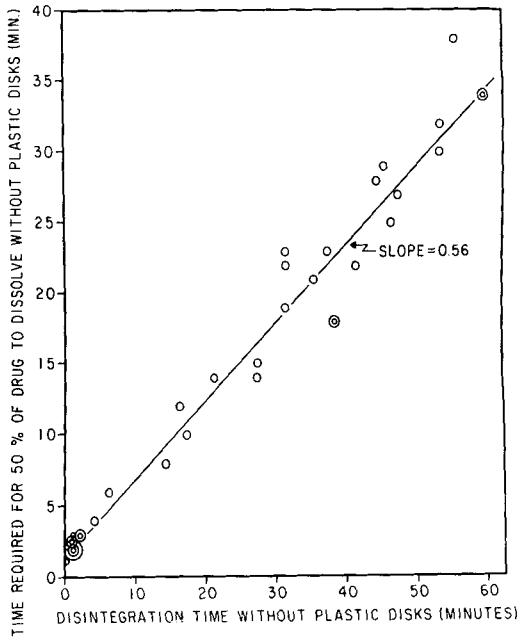


Fig. 11.—Correlation of time required for 50% of the drug to dissolve with disintegration time for tablets containing aspirin-phenacetin-caffeine. Each point represents a single tablet.

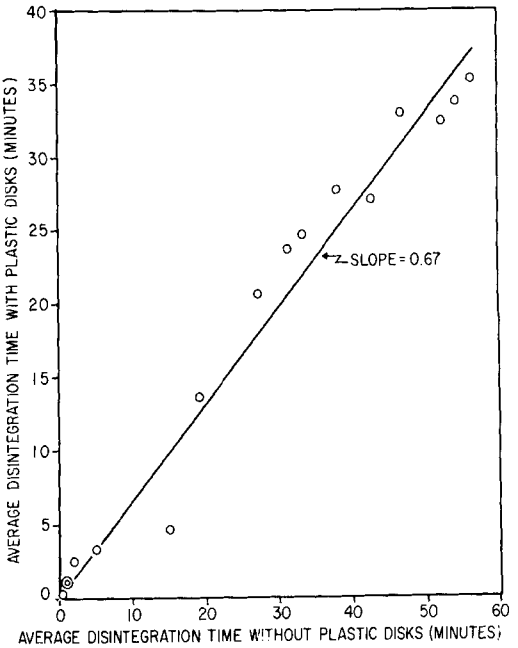


Fig. 12.—Correlation of average disintegration time determined with plastic disks with average disintegration time determined without plastic disks for tablets containing aspirin-phenacetin-caffeine.

p-aminosalicylate, Chapman, *et al.*, showed that results of disintegration tests obtained with rubber disks were significantly different ($P = 0.05$) than results obtained with plastic disks.

Tablets Containing Sodium *p*-Aminosalicylate.—The data of Chapman, Crisafio, and Campbell (9) were used. For five of the tablets studied in this series, the order of excretion rates of NaPAS in human subjects was A > D > E > F > G, whereas the order of *in vitro* dissolution rates reported was A > D > F > E > G. The order of per cent availability of NaPAS based on 8-, 24-, and 32-hr. excretion data was A ≃ D > E > F > G. As an index of the rates of dissolution *in vitro*, we have estimated the time required for 50% of the drug to dissolve ($t_{50\%}$) from their Fig. 2. Plots of the variables, per cent availability *vs.* $t_{50\%}$ and per cent availability *vs.* disintegration time with rubber disks, are shown in Fig. 14A. Plots of $t_{50\%}$ (obtained with method of Chapman, *et al.*) against disintegration time determined with method of Chapman, *et al.*, and with U.S.P. XV fluids plus plastic disks

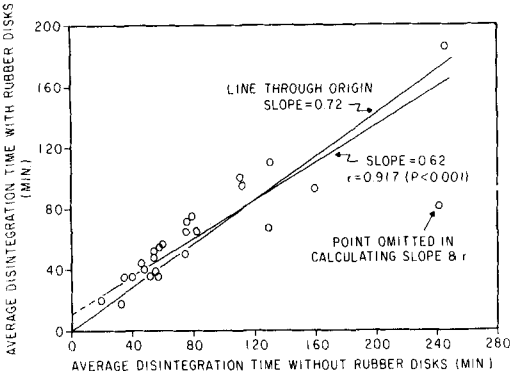


Fig. 13.—Data of Chapman, D. G., Crisafio, R., and Campbell, J. A., THIS JOURNAL, **43**, 297(1954), for sugar-coated tablets containing riboflavin.

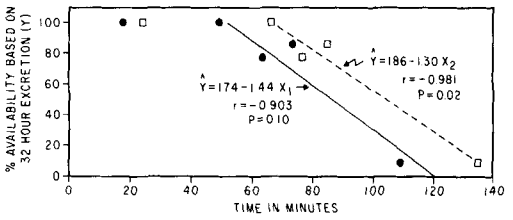


Fig. 14A.—A plot of per cent availability of sodium *p*-aminosalicylate based on 32-hr. urinary excretion against disintegration time in min.; data from Ref. 9.

TABLE VI.—SUMMARY OF SLOPES (s), INTERCEPTS (y_0), LINEAR CORRELATION COEFFICIENTS (r), NUMBER OF OBSERVATIONS (n), AND PROBABILITIES OF SIGNIFICANCE (P) FOR CORRELATIONS BETWEEN DISINTEGRATION TIMES WITH AND WITHOUT DISKS AND RATE OF DISSOLUTION

Ordinate (y) → / Abscissa (x) → / Tablet Series	Linear Regression: Average Disintegration Time with Disks *vs.* Average Disintegration Time without Disks	Linear Regression: Time Required for 50% of the Drug to Dissolve *vs.* Disintegration Time without Disks	Linear Regression: Time Required for 50% of the Drug to Dissolve *vs.* Disintegration Time with Disks
Anti-inflammatory steroid, lots A–L	Relationship curvilinear and not satisfactory for predictive purposes since too scattered	$s = 1.02$ $y_0 = -2.1$ $r = 0.969$ ($n = 14$) $p < 0.001$	No significant relationship
Sulfonamide lots A, B, D, E	Not studied	$s = 1.82$ $y_0 = -1.1$ $r = 0.942$ ($n = 8$) $P < 0.001$	Not studied
Sulfonamide lots, C, F, G	Not studied	$s = 1.10$ $y_0 = -3.6$ $r = 0.995$ ($n = 6$) $P < 0.001$	Not studied
Antidiabetic agent lots A–G	Not significant[a] $P > 0.10$	No significant relationship	No significant relationship[a]
Aspirin-phenacetin-caffeine combination	$s = 0.66$ (0.67) $y_0 = 0.54$ (0)	$s = 0.56$ $y_0 = +1.65$	$s = 0.82$ $y_0 = +1.65$
Lots A–P and granulation	$r = 0.987$ ($n = 17$) $P < 0.001$	$r = 0.984$ ($n = 35$) $P < 0.001$	$r = 0.959$ ($n = 35$) $P < 0.001$
Calculated from literature data			
Sugar-coated tablets containing riboflavin. data of Chapman, *et al.* (8)[b]	$s = 0.62$ (0.72) $y_0 = 11.5$ (0) $r = 0.917$ ($n = 26$) $p < 0.001$	...	...
Sugar-coated tablets containing sodium *p*-aminosalicylate, data of Chapman, *et al.* (9)	...	...	With Rubber Disks $s = 0.83$ $y_0 = -1.75$ $r = 0.995$ ($n = 5$) $p = 0.001$ With Plastic Disks $s = 0.48$ $y_0 = +16.0$ $r = 0.966$ ($n = 5$) $P < 0.001$

[a] It should be noted that with these tablets the disintegration times with and without disks were determined in different fluids. [b] Includes lots J through N and 1 through 22, with the exception of lot 17.

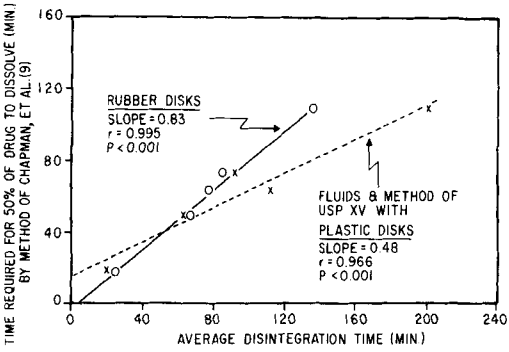


Fig. 14B.—A plot of time required for 50% of the drug to dissolve (determined by method of Chapman, *et al.*), against average disintegration time in min.; data from Ref. 9.

are shown in Fig. 14B. It is of interest that the correlation between $t_{50\%}$ and the disintegration method of Chapman (rubber disks with pH 1.6 and pH 8.0 fluids) gives a highly significant regression coefficient ($r = 0.995$). In this case the dissolution rate was determined during the same test as the disintegration time. One may conclude that the disintegration time determined by the method of Chapman, *et al.* (8, 9), was truly an indication of the rate of dissolution of the sodium *p*-aminosalicylate from the tablets. It is most probably for this reason that there was also a relationship between the per cent availability of the sodium *p*-aminosalicylate in human subjects and the disintegration time.

SUMMARY AND CONCLUSIONS

1. A total of 27 simultaneous dissolution-disintegration tests employing the U.S.P. tablet disintegration apparatus but without plastic disks were performed. These tests involved 12 lots of compressed tablets containing an anti-inflammatory steroid, seven lots of compressed tablets containing a sulfonamide, seven lots of compressed tablets containing an antidiabetic agent, and 16 lots of compressed tablets and one lot of granulation containing a combination of aspirin, phenacetin, and caffeine. A disintegration test employing the same apparatus but with plastic disks was also performed on all of these lots except those containing the sulfonamide.

2. The time required for 50% of the drug to dissolve ($t_{50\%}$) was estimated from each of the amount (or per cent) of drug in solution *vs.* time plots and used as a criterion of the rate of dissolution of the drugs from the tablets.

3. The existence or absence of a quantitative relationship between (*a*) $t_{50\%}$ and the results of the two types of disintegration tests, and (*b*) between results of the two types of disintegration tests were determined. Results are summarized in Table VI.

4. The results indicate there is an extreme specificity in the presence or absence of a relationship between rate of dissolution and disintegration time. In those cases where a quantitative relationship exists, the slopes of the "least squares" lines relating the variables were found to vary widely and depend upon the particular drug(s) involved, and in one case upon the presence or absence of a pharmaceutical adjuvant in the compressed tablet with the drug.

5. With two of the four series of tablets studied, the use of plastic disks in the tablet disintegration test was observed to mask real differences between the lots. In both of these series of tablets, one could not conclude that the tablets differed from lot-to-lot on the basis of their disintegration times determined with plastic disks. However, disintegration times determined without plastic disks and the $t_{50\%}$ values clearly indicated pronounced differences from lot-to-lot in each series. This indicates adoption of plastic disks in the official tablet disintegration test was perhaps hasty and not based upon enough experimental data.

6. These results raise questions as to the validity of uniform disintegration tests and disintegration time limits applied to large groups of drugs in compressed tablets. There will most probably be similar specificity of any correlation between either rate or extent of availability of drugs from tablets *in vivo* and either rate of dissolution or disintegration time determined by a particular test method. If this hypothesis is borne out by future experimentation, then limits of disintegration time or dissolution rate of individual drugs should be based on *in vivo-in vitro* correlations which, in turn, would be specific for the drug and the particular type or types of tablets to be controlled later by the *in vitro* test.

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Effect of Nicotine and Epinephrine on *in Vivo* Coagulation Time in Rabbits

By DUANE G. WENZEL and JASBIR SINGH

The intravenous administration of 0.01 or 0.02 mg./Kg. of nicotine to rabbits induced significant reductions in the time for blood to coagulate as measured by the Lee-White capillary tube method. Epinephrine induced a biphasic response with significant reductions resulting from small doses (0.01 and 0.02 mg./Kg.) and an increase from a larger dose (0.08 mg./Kg.). Intermediate doses (0.04 and 0.06 mg./Kg.) did not produce changes that were significantly different from control values. The combined treatment with nicotine, 0.01 mg./Kg., and epinephrine, 0.04 or 0.06 mg./Kg., increased the time to coagulate over that of the smaller doses of epinephrine or nicotine alone. The administration of piperoxan, 0.25 mg./Kg., 10 minutes before either nicotine or epinephrine, 0.01 mg./Kg., blocked the increased rate of coagulation.

Previous investigations have demonstrated that the chronic administration of nicotine to rabbits, in doses roughly comparable to the amount absorbed during smoking, produces a significant reduction in the blood coagulation time (1, 2). Because of the considerable epidemiological evidence relating smoking to the mortality from coronary heart disease, it is suggested that nicotine may serve as a precipitating factor through its effect upon the coagulation time.

Observations of the acute effects of nicotine upon blood coagulation have been few and generally contradictory. Takatsuki (3) reported that the subcutaneous administration of nicotine to rabbits in doses of from 0.5 to 5.0 mg./Kg. resulted in a decreased coagulation time. When he injected larger doses of from 10 to 30 mg./Kg. the coagulation times were then lengthened. The increase in rate as induced by the smaller quantities was found to be augmented by epinephrine and by cocaine, and either inhibited or reversed by yohimbine or ergotamine. From these findings Takatsuki concluded that the effect of nicotine on blood coagulation was dependent upon epinephrine release. In a later study, Kanowoka (4) administered nicotine intravenously to rabbits in doses of from 0.05 to 2 mg./Kg. and observed that the coagulation time was lengthened in a majority of the animals. The results, however, were inconsistent for a given dose and some animals responded to the treatment with a fall in the time to coagulate. The amount of nicotine employed was so large as to induce convulsions in most of the animals Doses of this magnitude, in addition to being unrelated to the amount of nicotine absorbed during smoking, are decidedly unphysiological and the extreme central stimulation may have induced secondary effects on the coagulation time.

The reported effects of smoking upon blood coagulation in humans have been at least as contradictory as those from animal studies. Grassi and Caltabiano (5) have presented evidence to indicate that smoking one cigarette results in an increased platelet count, shortened plasma coagulation and clot retraction times, and an augmentation of prothrombin activity and utilization. These findings are in accord with those of Eisen (6) who determined that smoking three to four cigarettes after a month's abstinence results in a coagulation time reduction to about one-half of the presmoking level. Blackburn, Orma, Härtel, and Punsar (7), on the other hand, were unable to demonstrate a significant effect of smoking upon blood coagulation as measured by the recalcified plasma Stypven time.

The primary purpose of this study was to reexamine the effect and the mechanism of action of acute nicotine administration upon *in vivo* blood coagulation. In order to establish the relationship of the nicotine effects to epinephrine release, actions of the latter agent were also examined. Despite the fact that epinephrine's role in blood coagulation has been studied by a number of investigators, there is still some confusion regarding its effects. That epinephrine influenced the coagulation time was first recognized by Vosburgh and Richards in 1903 (8). This work could not be confirmed by Wiggers (9), but was later verified by Van den Velden (10). It was in 1914 that Cannon and Gray (11) performed their classical studies indicating that epinephrine in small doses (0.001 mg./Kg.) shortened the coagulation time, but in larger doses (0.03 mg./Kg.) lengthened it. This experiment and many others which followed were performed with the coagulometer of Cannon and

Received December 18, 1961, from the Department of Pharmacology, The University of Kansas, School of Pharmacy, Lawrence.
Accepted for publication January 3, 1962.
Supported by a grant from the Life Insurance Medical Research Fund.

Mendenhall (12). The preceding observations regarding the clot accelerating effect of epinephrine have been found, by Forwell and Ingram (13), to be reproducible in anesthetized dogs or cats when the coagulometer principle was employed. The results could not be verified, however, under the same conditions by the standard Lee-White test tube method. For reasons that are not apparent, the administration of epinephrine to humans by these same investigators resulted in a shortened time when measured by the Lee-White tests. Furthermore, a significant fall in the time to coagulate with humans was observed only when silicon-coated tubes were employed. From this and additional evidence, Forwell and Ingram suggested that the action of epinephrine, at least in part, results from an increase in factor V activity. It was also suggested that the epinephrine effect may be duplicated by the unpleasant stimulus of blood withdrawal. Härtel (14), employing the plasma Stypven time previously found to be unaffected by smoking (7), observed that the subcutaneous injection of 0.7 mg. of adrenaline in humans caused a significant shortening. In this study all apparatus was siliconized.

As previous studies of the chronic effects of nicotine on blood coagulation had been satisfactorily performed using uncoated capillary tubes and with unanesthetized animals (1, 2), it was accordingly decided to determine the effects of nicotine and epinephrine in the same manner for this experiment.

EXPERIMENTAL

Six unanesthetized, adult, female, albino rabbits were employed for each drug treatment. To determine the mean coagulation times, three replications were made with each animal at each test period. Control mean clotting times were measured for each group immediately prior to the administration of the test agent. Blood was obtained from a puncture wound in the tail. After lightly and quickly blotting the first few drops of blood, an uncoated capillary tube with an internal diameter of approximately 1 mm. was used to collect blood to a length of about 3 cm. Capillary tubes were thoroughly washed with distilled water and dried before being employed. The end of the tube containing the blood first withdrawn was repeatedly broken off in small sections at a speed as great as was consistent with the observation of the fibrin thread. It was found that this could be accurately performed once in about every 2.5 seconds. Time was measured by a foot-actuated stopwatch.

Following the control coagulation time determinations and the administration of the appropriate drug dose, the measurements were repeated at 1 minute, 15 minutes, and every 15 minutes thereafter until the time to coagulate had returned to within 1 to 2 seconds difference from that of the control value. For some of the treatments, testing was continued beyond this point in order to demonstrate the absence of any delayed action. As three replications were performed at each period tested, the first determination was started shortly before the indicated time and the last one shortly thereafter. Each mean coagulation time therefore represents three determinations upon each of six animals for a total of 18 individual values. The *t*-tests and standard errors of the mean were calculated on the basis of six mean values for each test.

Drugs were administered in normal saline with a total volume of 0.25 ml. It has been suggested that, by what may be a stress response, repeated venepuncture alone may induce a reduction in the coagulation time. The injection of 0.25 ml. of saline followed by repeated blood collections did not produce a significant change in the time at any of the periods tested.

The following drug doses and combinations of drugs expressed as mg. drug per Kg. body weight were tested for their effect upon the coagulation time: nicotine 0.01 and 0.02; epinephrine 0.01, 0.02, 0.04, 0.06, and 0.08; nicotine 0.01 and epinephrine 0.04; nicotine 0.01 and epinephrine 0.06; piperoxan 0.25; piperoxan 0.25 and nicotine 0.01; and piperoxan 0.25 and epinephrine 0.01. Administration of the piperoxan preceded that of the

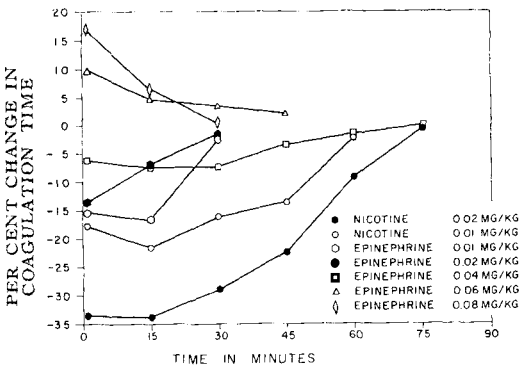


Fig. 1.—Effects of several doses of nicotine and epinephrine upon the mean blood coagulation time in rabbits. The first reading was obtained at 1 minute.

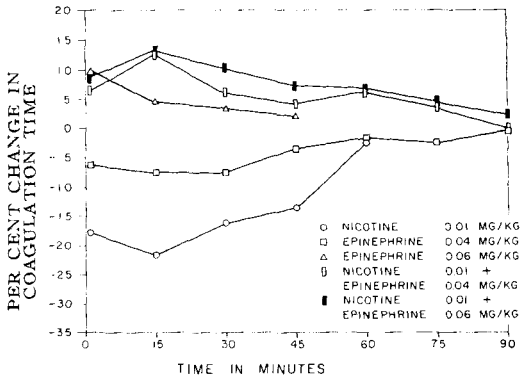


Fig. 2.—Effects of the combined administration of nicotine and epinephrine upon the mean blood coagulation time of rabbits. The first reading was obtained at 1 minute.

TABLE I.—COAGULATION TIMES IN RABBITS TREATED WITH NICOTINE AND EPINEPHRINE[a]

Treatment, mg./Kg.	Coagulation Times, sec. ± S.E. Initial Time	1 Min. After Treatment	% Change	P
Saline	28.8 ± 1.3	28.4 ± 1.6	−1.4	>0.50
Nicotine 0.01	37.4 ± 1.1	30.7 ± 1.0	−17.9	<0.01[b]
Nicotine 0.02	34.7 ± 0.34	23.1 ± 0.67	−33.4	<0.001[b]
Epinephrine 0.01	28.9 ± 1.3	24.2 ± 1.1	−16.3	<0.02[b]
Epinephrine 0.02	30.9 ± 0.86	26.7 ± 1.27	−13.6	<0.02[b]
Epinephrine 0.04	31.0 ± 1.4	28.9 ± 1.9	−7.0	>0.05
Epinephrine 0.06	33.1 ± 2.2	36.4 ± 1.3	+9.1	>0.05
Epinephrine 0.08	30.1 ± 0.75	34.5 ± 1.5	+12.8	<0.02[b]
Nicotine 0.01, epinephrine 0.04	29.4 ± 1.2	31.4 ± 1.2	+6.4	>0.05
Nicotine 0.01, epinephrine 0.06	30.9 ± 1.88	33.4 ± 1.89	+7.5	>0.05
Piperoxan 0.25	31.7 ± 0.89	29.6 ± 0.85	+6.6	>0.05
Piperoxan 0.25, epinephrine 0.01	31.2 ± 1.87	30.0 ± 2.08	−3.8	>0.05
Piperoxan 0.25, nicotine 0.01	29.4 ± 1.58	29.4 ± 1.25	0	>0.05

[a] Coagulation times were measured using the Lee-White capillary tube method. Six animals were employed for each treatment tested with three replications at each period of testing. [b] Significant values.

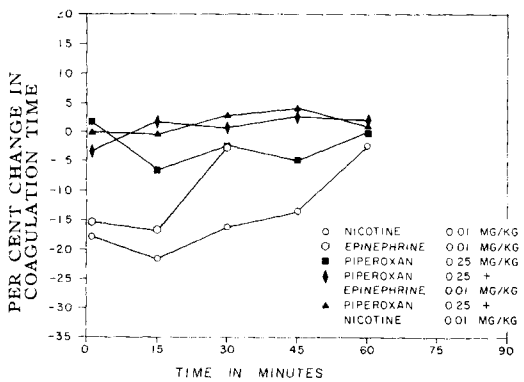


Fig. 3.—The effect of piperoxan upon the mean blood coagulation response of rabbits to nicotine and epinephrine. The first reading was obtained at 1 minute.

nicotine or epinephrine by 10 minutes in order to allow adequate time for the development of adrenergic blockade. The times at which the rate of clotting was measured for the combined treatment with piperoxan were relative to the time of nicotine or epinephrine administration.

RESULTS AND DISCUSSION

The mean initial coagulation times (controls), the times at the 1-minute period, their standard errors, the per cent change in coagulation time during this period, and the level of significance of the difference between the means are given in Table I. As the greatest drug-induced effects, with few exceptions, were apparent at the 1-minute test period, these mean experimental values were compared by the *t*-test to those of the corresponding controls. The results are treated graphically in Figs. 1, 2, and 3 to present the time-related pattern of the response.

Separate Nicotine and Epinephrine Effects.—Doses of nicotine were selected so as to induce changes in the coagulation time without the convulsive seizures reported by previous investigators (3, 4). Initial exploratory experiments indicated that the intravenous administration of 0.03 or 0.04 mg./Kg. of nicotine induced tremors in many of the animals tested. It was further determined that neither nicotine nor epinephrine in intravenous doses up to 0.02 and 0.08 mg./Kg., respectively, would cause excessive stimulation with the exception of occasional animals which responded with respiratory stimulation. These doses were then selected as the arbitrary limits to be employed.

From the values in Table I and the points in Fig. 1, it is apparent that nicotine at both of the doses tested significantly reduced the time required for blood to coagulate ($P < 0.01$ and 0.001). It seems likely that the intravenous doses of 0.01 and 0.02 mg./Kg. as employed in this study are comparable to larger subcutaneous doses reported to induce a similar effect (3). Others (4) have noted that the intravenous injection of larger doses (0.5 to 2mg./Kg.) of nicotine results in a lengthened time to coagulate, although some of the animals tested responded with reduction in the time. Such variance was not encountered with the smaller doses used in this study as all animals, without exception, responded by an increased rate of coagulation.

Epinephrine in doses of 0.01 and 0.02 mg./Kg. also significantly reduced the coagulation times ($P < 0.02$). The response to these doses was strikingly similar except for a longer significant reduction with the larger dose. Doses of 0.04 and 0.06 mg./Kg. induced a fall and an increase, respectively, in the times to coagulate. While these differences were not significant in the statistical sense, from an examination of the time-effect curves in Fig. 1 it appears unlikely that the changes were not attributable to the treatments involved. As the largest dose of the epinephrine 0.08 mg./Kg. resulted in a significant extension of the coagulation time ($P < 0.02$), it is apparent that the results of the epinephrine treatment are in agreement with those of Cannon and Gray (11) who first demonstrated the biphasic response. The data provide no explanation for the inability of Forwell and Ingram to observe an effect upon coagulation time in animals when using the Lee-White test tube method

(13). While the capillary method was employed, the tubes were uncoated and the animals were unanesthetized which, if anything, would be expected to mask the effect of epinephrine rather than to increase the sensitivity.

Combined Nicotine-Epinephrine Effects.—The results obtained by combining 0.01 mg./Kg. of nicotine with either 0.04 or 0.06 mg./Kg. of epinephrine are shown in Fig. 2 and Table I. Changes in both instances were in the direction of an increased time to coagulate, although both drugs individually had depressed the coagulation time. It is perhaps worthy of note that the combined nicotine-epinephrine, while not inducing a significant increase in the coagulation time as measured by the *t*-test, apparently prolonged the duration of the response. Further, the direction of the change is what would be anticipated if nicotine exerts its action through catecholamine release.

Modification of Nicotine and Epinephrine Responses by Piperoxan.—The injection of piperoxan alone in a dose of 0.25 mg./Kg. caused a minimal and nonsignificant increase in the rate of coagulation (Table I and Fig. 3). Administration of this dose 10 minutes prior to either 0.01 mg./Kg. of epinephrine or nicotine resulted in coagulation times which varied no more than did repeated control determinations. This blockade of the nicotine effect is consistent with the results of Takatsuki (3), demonstrating that nicotine exerts its characteristic action on the coagulation time through the release of catecholamines. As the actions of nicotine and epinephrine are both apparently mediated through the latter agent, and as the response to their combined administration indicates an additive effect, it is likely that the combination of smaller doses of each would result in a shorter coagulation time. Although the experimental evidence does not directly support the contention, it is further likely that the amounts of nicotine absorbed during smoking would increase rather than decrease the rate of coagulation, even in the presence of systemic catecholamine release. In man, Johnston has reported that 0.08 to 0.13 mg. of nicotine intravenously produces effects similar to those induced by one deep inhalation of cigarette smoke (15).

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Use of 3-Azabicyclo[3.2.2.]nonane in the Mannich Reaction I

Substituted β-Amino Ketones

By C. DeWITT BLANTON, Jr., and W. LEWIS NOBLES

A group of Mannich bases, indicated as types I, II, and III, have been prepared utilizing 3-azabicyclo[3.2.2.]nonane as the amine component. These β-aminoketones are to be screened for possible pharmacodynamic activity.

RECORDED in the literature (1–11) are numerous ketonic Mannich bases, prepared for pharmacological testing as antispasmodics, analgesics, chemotherapeutic agents, and local anesthetics. Such compounds may, in general, be prepared easily by means of the Mannich reaction which utilizes the appropriate ketone, formaldehyde, or paraformaldehyde, and the desired amine. This may be illustrated as follows

$$RCOCH_3 + HCHO + R'_2NH \cdot HCl \rightarrow RCOCH_2CH_2NR'_2 \cdot HCl + H_2O$$

Received October 23, 1961, from the School of Pharmacy, The University of Mississippi, University.
Accepted for publication November 24, 1961.

The rather extensive literature dealing with this reaction has been reviewed by Blicke (12), Karbe (13), Reichert (14), and more recently by Hellmann and Opitz (15).

The great versatility of these ketonic Mannich bases prompted us to prepare a number of Mannich bases of types I, II, and III, in which the amine moiety is 3-azabicyclo[3.2.2.]nonane. In addition, the concept of vinylogous substances, as set forth by Fuson (16), has led us to prepare several vinylogs of types I and II. (See Tables I–III.)

To study more fully the effect of structural variants, Mannich bases have been prepared of

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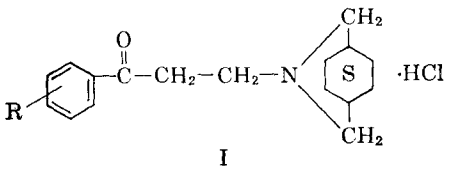
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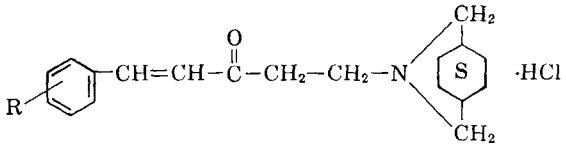
To study more fully the effect of structural variants, Mannich bases have been prepared of

TABLE I.—MANNICH BASES TYPE I



I

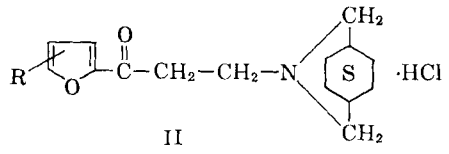
No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	Hydrogen[d]	66.0	199–200	$C_{17}H_{23}NO \cdot HCl$	67.47	66.83	8.33	8.38	4.77	4.77
2	*p*-Nitro[d]	41.4	202–203	$C_{17}H_{22}N_2O_3 \cdot HCl$	58.70	58.26	6.95	6.94	8.05	8.06
3	*p*-Chloro[d]	45.7	207–208	$C_{17}H_{22}ClNO \cdot HCl$	60.54	60.53	7.17	7.16	4.15	4.17
4	*p*-Methoxy	51.6	213–214	$C_{18}H_{25}NO_2 \cdot HCl$	66.76	66.78	8.04	8.25	4.33	3.98
5	*p*-Ethoxy	50.2	201–201	$C_{19}H_{27}NO_2 \cdot HCl$	67.54	67.64	8.35	8.41	4.15	4.45
6	*o*-Hydroxy	36.5	196–197	$C_{17}H_{23}NO_2 \cdot HCl$	65.91	65.21	7.75	7.68	4.52	4.56
7	*p*-Methyl	54.5	220–221	$C_{18}H_{25}NO \cdot HCl$	70.22	69.88	8.51	8.52	4.45	4.48
8	*p*-Fluoro[d]	35.4	203–204	$C_{17}H_{22}FNO \cdot HCl$	63.62	63.67	7.53	7.69	4.36	4.53
9	*p*-Bromo	33.2	211–212	$C_{17}H_{22}BrNO \cdot HCl$	54.78	53.67	6.22	5.99	3.76	3.77
10	*p*-Hydroxy	38.4	226–228	$C_{17}H_{23}NO_2 \cdot HCl$	65.90	65.65	7.81	7.99	4.52	4.59
11	*p*-Phenyl	53.7	211–212	$C_{23}H_{27}NO \cdot HCl$	74.69	74.53	7.58	7.56	3.78	3.95
12	*m*-Nitro	21.3	230–231	$C_{17}H_{22}N_2O_3 \cdot HCl$	60.26	60.29	6.84	7.18	8.27	8.48
13	*p*-Ethyl	53.4	211–213	$C_{19}H_{27}NO \cdot HCl$	70.89	71.01	8.77	8.35	4.35	4.44
14	*m*-Bromo	32.7	217–218	$C_{17}H_{22}NO \cdot HCl$	54.65	54.39	6.17	5.94	3.76	3.66
15	*m*-Hydroxy	48.0	210–212	$C_{17}H_{23}NO_2 \cdot HCl$	65.90	65.88	7.81	7.83	4.52	4.66
16	*p*-Amino	49.5	99–100	$C_{17}H_{24}N_2O$	75.00	74.69	8.82	9.08	10.29	10.45



17	Hydrogen	61.8	203–204	$C_{19}H_{25}NO \cdot HCl$	71.34	70.78	8.19	8.16	4.38	4.37
18	*p*-Chloro	...	217–218	$C_{19}H_{24}ClNO \cdot HCl$	64.41	64.69	7.11	7.03	3.95	3.84
19	*p*-Methoxy	43.3	212–213	$C_{20}H_{27}NO_2 \cdot HCl$	68.65	68.65	8.07	8.17	4.00	4.31

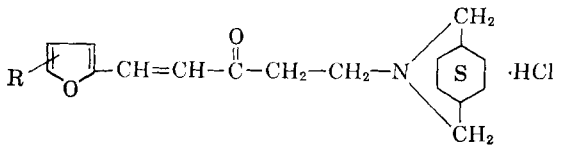
[a] All Mannich bases in this table were recrystallized from an ethanol-acetone solution, ethanol, or ethanol-water solution. Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for one-half mole of water.

TABLE II.—MANNICH BASES TYPE II



II

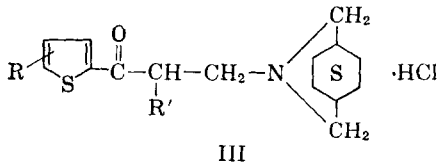
No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	5-Nitro	30.5	212–214	$C_{15}H_{20}N_2O_4 \cdot HCl$	54.80	54.58	6.40	6.59	8.50	8.56



2	Hydrogen[d]	12.6	198–199	$C_{17}H_{23}NO_2 \cdot HCl$	64.96	64.80	7.86	8.20	4.46	4.50
3	5-Nitro	22.6	185–188	$C_{17}H_{22}N_2O_4 \cdot HCl$	57.54	57.67	6.49	6.63	7.89	7.97

[a] All Mannich bases in this table were recrystallized from an ethanol-acetone, ethanol, or ethanol-water solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig, Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for one-fourth mole of water.

TABLE III.—MANNICH BASES TYPE III

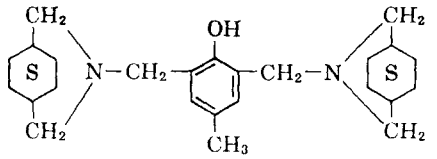
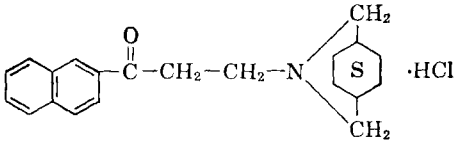


III

No.[a]	R	R′	Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	Hydrogen	Hydrogen	58.5	203–204	$C_{15}H_{21}NOS \cdot HCl$	60.10	59.94	7.34	7.44	4.67	4.61
2	Hydrogen	Methyl	37.9	200–201	$C_{16}H_{23}NOS \cdot HCl$	61.21	60.91	7.66	7.49	4.46	4.12
3	5-Bromo	Methyl	39.4	200–201	$C_{16}H_{22}BrNOS \cdot HCl$	48.92	48.87	5.90	5.83	3.57	3.33

[a] All Mannich bases in this table were recrystallized from an ethanol-acetone, ethanol, or ethanol-water solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

TABLE IV.—MISCELLANEOUS MANNICH BASES

No.[a]		Yield, %	M.p.,[b] °C.	Formula	Analyses,[c] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
										
1	...	89.9	95–100	$C_{25}H_{38}N_2O$	78.48	78.09	10.01	9.60	...	...
										
2	2-Naphthyl	42.0	221–222	$C_{21}H_{25}NO \cdot HCl$	73.34	73.32	7.62	7.53	4.07	4.23

[a] All Mannich bases in this table were recrystallized from an ethanol-acetone solution, ethanol, or ethanol-water solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

2-acetonaphthone and phenolic compounds, *viz.*, the disubstituted product of *p*-cresol (see Table IV). The change in activity with variation in chemical structure in other chemical series led us to study additional transformations. Denton and his co-workers (4) indicated that the reduction of the piperidino Mannich bases to the corresponding aminoalcohols led to a marked increase in antispasmodic activity in all cases except those involving a thiophene derivative. Also, many of the tertiary alcohols, prepared by these workers by the addition of a Grignard reagent to the Mannich base, exhibited increased activity over that of the parent ketone. Certain of these suggested modifications have been incorporated into the structures presented here and these studies, along with the results of pharmacological screening, will be reported in subsequent publications. Preliminary evaluation of compound No. 1 from Table II has been obtained.[1] Oral administration of 300 mg./Kg. of this compound to a rat (Pratt) failed to produce any overt activity.

EXPERIMENTAL

Experimental Results.—β-[3-(3-Azabicyclo-[3.2.2.]nonyl)]-4-aminopropiophenone was prepared according to the method of Wright and Freifelder (17). *p*-Chlorobenzalacetone was synthesized according to the method previously outlined (18). 2,6-di-[3-(3-Azabicyclo[3.2.2.]nonyl)-methyl]-*p*-cresol was prepared according to the method of Bruson and MacMullen (19). 2-Propionylthiophene was synthesized by the procedure of Kosak and Hartough (20), employing propionic anhydride rather than acetic anhydride. 2-Propionyl-5-bromothiophene was prepared according to the method of Hartough and Conley (21). This procedure was

[1] The authors wish to thank Dr. Paul Craig of Smith Kline & French Laboratories for arranging for the pharmacological testing.

modified by substituting 2-bromothiophene for 2-chlorothiophene and propionic anhydride for acetic anhydride. 3-Azabicyclo[3.2.2.]nonane was supplied through the courtesy of Eastman Chemical Products, Inc. All other ketones in this study were commercially available.

The Mannich reaction was carried out as previously described (8), utilizing procedure B. A solid usually appeared within 30–90 minutes and acetone was added to complete precipitation. It was observed that some of these compounds were only slightly soluble in ethanol, especially the ones of more complex structure. In such cases a few drops of water were added to help facilitate solution in the recrystallization processes. In general, the reactions proceeded smoothly to yield the expected product. It may be noted, however, that excellent yields are seldom obtained in the Mannich reaction due to the complexity of the products obtained which may be occasioned by by-product formation. The furan derivatives consistently produced low yields.

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Sympathetic Nervous System Blocking Agents

Investigation of Ethyl-, Hydroxyethyl-, Vinyloxyethyl-, and Propargyl-benzyldimethylammonium Halides and Related Compounds

By JAMES H. SHORT and URSULA BIERMACHER

A series of quaternary benzylamine derivatives has been prepared. They were examined for their ability to block the sympathetic nervous system without also blocking the parasympathetic system. The most active compounds were derivatives of benzyl(vinyloxyethyl)dimethylammonium halide. The *o*-bromo analog has activity of the same order of magnitude as bretylium. Pharmacological results and structure-activity relationships are discussed.

THROUGH routine screening of compounds for cardiovascular effects, it was observed that benzyl(vinyloxyethyl)dimethylammonium bromide (Table I, No. 1) had a blocking effect on the sympathetic nervous system, but apparently had little or no effect on the parasympathetic system. It appeared, therefore, to have activity of the same type as bretylium, which has recently been investigated in the clinic as an antihypertensive agent (1).

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The required intermediate amines and halides were prepared by standard methods and are described in the experimental section.

PHARMACOLOGICAL RESULTS[1]

The effectiveness of these compounds as sympathetic blocking agents was determined in unanesthetized cats. The candidate drugs were administered orally, and the degree and duration of the prolapse of the nictitating membrane were the criteria used to determine whether or not the desired activity was present. Since parasympatho-

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modified by substituting 2-bromothiophene for 2-chlorothiophene and propionic anhydride for acetic anhydride. 3-Azabicyclo[3.2.2.]nonane was supplied through the courtesy of Eastman Chemical Products, Inc. All other ketones in this study were commercially available.

The Mannich reaction was carried out as previously described (8), utilizing procedure B. A solid usually appeared within 30–90 minutes and acetone was added to complete precipitation. It was observed that some of these compounds were only slightly soluble in ethanol, especially the ones of more complex structure. In such cases a few drops of water were added to help facilitate solution in the recrystallization processes. In general, the reactions proceeded smoothly to yield the expected product. It may be noted, however, that excellent yields are seldom obtained in the Mannich reaction due to the complexity of the products obtained which may be occasioned by by-product formation. The furan derivatives consistently produced low yields.

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Sympathetic Nervous System Blocking Agents

Investigation of Ethyl-, Hydroxyethyl-, Vinyloxyethyl-, and Propargyl-benzyldimethylammonium Halides and Related Compounds

By JAMES H. SHORT and URSULA BIERMACHER

A series of quaternary benzylamine derivatives has been prepared. They were examined for their ability to block the sympathetic nervous system without also blocking the parasympathetic system. The most active compounds were derivatives of benzyl(vinyloxyethyl)dimethylammonium halide. The *o*-bromo analog has activity of the same order of magnitude as bretylium. Pharmacological results and structure-activity relationships are discussed.

THROUGH routine screening of compounds for cardiovascular effects, it was observed that benzyl(vinyloxyethyl)dimethylammonium bromide (Table I, No. 1) had a blocking effect on the sympathetic nervous system, but apparently had little or no effect on the parasympathetic system. It appeared, therefore, to have activity of the same type as bretylium, which has recently been investigated in the clinic as an antihypertensive agent (1).

Interest in this type of pharmacological activity led us to synthesize a series of quaternary salts in order to investigate structure-activity relationships. Some of these compounds were prepared by the reaction of a benzyl halide with a tertiary amine. For those desired compounds which were not accessible by this route, the alternative procedure, reaction of an N,N-dimethylbenzylamine with an alkyl halide was employed.

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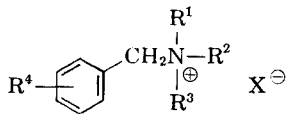
PHARMACOLOGICAL RESULTS[1]

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TABLE I.—QUATERNARY SALTS OF SUBSTITUTED BENZYLAMINES



No.	R^4	R^1	R^2	R^3	X	M.p., °C.	Method[a]	Formula	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
1	H	$CH_2CH_2OCH{=}CH_2$	CH_3	CH_3	Br	141–142	B[b]	$C_{13}H_{20}BrNO$	54.55	7.04	...	54.96	7.04	...
2	2-F	$CH_2CH_2OCH{=}CH_2$	CH_3	CH_3	Cl	149–149.5	B[b]	$C_{12}H_{19}ClFNO$	60.10	7.38	5.40	60.23	7.49	5.24
3	2-Cl	$CH_2CH_2OCH{=}CH_2$	CH_3	CH_3	I	128.5–129	[c]	$C_{13}H_{19}ClINO$	42.46	5.21	3.81	42.59	5.23	3.60
4	2-Br	$CH_2CH_2OCH{=}CH_2$	CH_3	CH_3	Br	117–118	B[d]	$C_{13}H_{19}Br_2NO$	42.76	5.25	3.84	43.01	5.07	3.61
5	2-CF_3	$CH_2CH_2OCH{=}CH_2$	CH_3	CH_3	Br	59.5–62	B	$C_{14}H_{19}BrF_3NO$[m]	...	...	3.95	...	...	3.78
6	2-Br	$CH_2CH_2OCH{=}CH_2$		 [e]	Br	105–106.5	B[f]	$C_{15}H_{23}Br_2NO$	47.43	5.72	3.45	47.52	5.62	3.39
7	H	C_2H_5	CH_3	CH_3	I	120–121	A[f]	$C_{11}H_{18}IN$	45.37	6.23	4.81	45.68	6.43	4.61
8	2-OCH_3	C_2H_5	CH_3	CH_3	I	108–109	A[f]	$C_{12}H_{20}INO$	44.87	6.27	4.36	44.88	6.43	4.22
9	2-Cl	C_2H_5	CH_3	CH_3	I	130.5–131.5	A[f]	$C_{11}H_{17}ClIN$	40.57	5.26	4.30	40.66	5.43	4.42
10	4-Cl	C_2H_5	CH_3	CH_3	I	160.5–161.5	A[f]	$C_{11}H_{17}ClIN$	40.57	5.26	4.30	40.70	5.10	4.21
11	2,4-Di-Cl	C_2H_5	CH_3	CH_3	I	166.5–167.5	A[f]	$C_{11}H_{16}Cl_2IN$	36.69	4.48	3.89	36.63	4.47	3.75
12	4-Br	C_2H_5	CH_3	CH_3	I	159.5–160.5	A[g]	$C_{11}H_{17}BrIN$	35.70	4.62	3.78	35.83	4.76	3.70
13	4-Cl	CH_3		 [l]	Cl	201.5–202	B[g]	$C_{12}H_{17}Cl_2NO$	54.98	6.53	5.34	54.75	6.24	5.16
14	2-Br	CH_2CH_2OH	C_2H_5	C_2H_5	Br	119.5–120.5	B[g]	$C_{13}H_{21}Br_2NO$	42.53	5.76	3.82	42.30	5.98	3.77
15	2-Br	C_2H_5	CH_2CH_2OH	CH_2CH_2OH	Br	110.5–112	B[g]	$C_{13}H_{21}Br_2NO_2$	40.75	5.53	3.65	40.45	5.56	3.70
16	2,4-Di-Cl	CH_3	CH_2CH_2OH	CH_2CH_2OH	Cl	113[h]	B[i]	$C_{12}H_{18}Cl_3NO_2$	45.81	5.77	4.45	45.68	5.68	4.43
17	2,4-Di-Cl	$CH_2CHOHCH_3$	CH_3	CH_3	Cl	90–92	B	$C_{12}H_{18}Cl_3NO$	48.27	6.07	4.69	48.05	6.26	4.49
18	4-Cl	CH_2CH_2OH	C_6H_{13}-*n*	C_6H_{13}-*n*	Cl	113.5–115	B	$C_{21}H_{37}Cl_2NO$	64.21	9.55	3.58	64.60	9.87	3.57
19	2-Cl	CH_2CH_2OH	CH_2CH_2OH	CH_2CH_2OH	Cl	134.5–136.5	B[g]	$C_{13}H_{21}Cl_2NO_3$	50.33	6.82	4.52	50.11	6.73	4.30
20	2-Cl	$CH_2CH_2CH_2OH$	C_2H_5	C_2H_5	Cl	144.5–145.5	B[i]	$C_{14}H_{23}Cl_2NO$	57.54	7.93	4.79	57.59	7.97	4.60
21	2-Cl	C_4H_9-*n*	CH_2CH_2OH	CH_2CH_2OH	Cl	103–106	B	$C_{15}H_{25}Cl_2NO_2$	55.91	7.82	4.34	55.71	7.50	4.45
22	H	$CH_2C{\equiv}CH$	CH_3	CH_3	Br	156–157	A[g]	$C_{12}H_{16}BrN$	56.71	6.34	5.50	56.63	6.53	5.54
23	3,4-Di-OCH_3	CH_3	$CH_2C{\equiv}CH$	$CH_2C{\equiv}CH$	Br	192.5–193.5	A[i]	$C_{16}H_{20}BrNO_2$	56.82	5.96	4.14	56.72	6.15	4.12
24	2-Br[k]	C_2H_5	CH_3	CH_3	I	119.5–120	A[f]	$C_{11}H_{17}BrIN$	35.69	4.63	3.78	35.94	4.69	3.60

[a] Method A is the reaction of a benzylamine with an alkyl halide; method B is the reaction of a benzyl halide with an alkylamine. [b] Recrystallized from methyl ethyl ketone containing a little methanol. [c] For method of preparation, see Experimental section. [d] Recrystallized from acetone. [e] NR^2R^3 = piperidino. [f] Recrystallized from methyl ethyl ketone. [g] Recrystallized from 2-propanol. [h] Melting point was not sharp. [i] Recrystallized from 2-propanol-ethyl ether. [j] Prepared by Miss Carol Christensen from N-methylveratrylamine and propargyl bromide, and recrystallized from ethanol-ether. [k] Bretylium iodide. [l] NR^2R^3 = morpholino. [m] Br: Calcd.: 22.57; Found: 22.56.

TABLE II.—MISCELLANEOUS COMPOUNDS

$$R^4\text{—}N^{\oplus}(R^1)(R^2)(R^3)\ X^{\ominus}$$

No.	R	R^2	R^3	R^4	X	M.p., °C.	Formula	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
25	...	a	CH_3	CH_3	I	260–262[b]	$C_{11}H_{24}IN$	44.45	8.14	4.72	44.43	8.27	4.76
26	...	a	CH_3	$CH_2C_6H_5$	Br	171	$C_{17}H_{28}BrN$	62.57	8.65	4.29	62.41	8.39	4.30
27	1-Bromo-2-naphthylmethyl	CH_3	CH_3	C_2H_5	I	147.5–149	$C_{15}H_{19}BrIN$	42.89	4.55	3.33	42.61	4.83	3.34
28	2-Furylmethyl	CH_3	CH_3	C_2H_5	I	104–105[c]	$C_9H_{16}INO$	38.45	5.73	4.98	38.49	5.55	5.09
29	2-Furylmethyl	CH_3	CH_2	*o*-Bromobenzyl	Br	134–135.5	$C_{14}H_{17}Br_2NO$	44.82	4.57	3.73	44.80	4.76	3.63

[a] R^1R^2N = 2,2,4,6-tetramethylpiperidino. [b] Recrystallized from acetone. [c] Recorded m.p. is 103–104° (9).

lytic agents and ganglionic blocking agents alter the pupillary responses of the eye, normal responses were taken as indications that the parasympathetic nervous system was not also being blocked.

When the candidate drugs failed to cause a prolapse of the nictitating membrane at an initial low dose level, dosage was increased as high as 30 mg./Kg. Those substances which failed to show a response at such a high dose were classified as inactive. The active compounds are compared in Table III.

Several compounds related to bretylium, but bearing different substituents on the benzene ring, were examined. It was found that when the benzene ring carried a substituent in the *para* position (Table I, Nos. 10, 11, 12) the desired activity was not observed. However, when the bromine atom in the *ortho* position of the benzene ring was replaced by hydrogen, methoxy, or chloro (Nos. 7, 8, 9) these compounds were still active, but less so than bretylium (see Table III).

The compounds obtained by replacing the *o*-bromobenzyl moiety of bretylium with the 1-bromo-2-naphthylmethyl or 2-furylmethyl groups were inactive.

The quaternary salts prepared from various ethanol- and propanolamines were uniformly inactive (Table I, Nos. 14–21).

The most active group of compounds were those quaternary salts derived from (vinyloxyethyl)-dimethylamine (Table I, Nos. 1–5). Of this group, the *o*-bromo derivative (No. 4) was outstanding. It appears to be as potent as bretylium (No. 24), but with longer duration of activity. It is interesting to note that when the dimethylamino group of No. 4 is replaced by the piperidine group (No. 6), activity is lost.

The analogs of Nos. 4 and 24 containing no substituent on the benzene ring (Nos. 1 and 7) have comparable activity, as do the *o*-chloro analogs (Nos. 3 and 9).

Acute toxicity studies were carried out only with Nos. 1, 4, and 24. They were administered orally to mice, and all three failed to cause death at doses of 500 mg./Kg.

The activity shown by 1,1,2,2,4,6-hexamethylpiperidinium iodide (Table II, No. 25) appears to be due to ganglionic blockade rather than the desired activity.

EXPERIMENTAL[2]

***o*-Bromobenzyl Bromide.**—The procedure of DeTar and Carpino (2) was modified by running the reaction at 150° in the absence of benzoyl peroxide for 3 hours. The colorless oil boiled at 129–130° (15 mm.), n_D^{21} 1.6195.

1-Bromo-2-bromomethylnaphthalene.—A mixture of 53 Gm. (0.3 mole) of N-bromosuccinimide and 66.3 Gm. (0.3 mole) of 1-bromo-2-methylnaphthalene (Aldrich Chemical Co.) in 700 ml. of carbon tetrachloride was illuminated from below by a 150-watt light bulb. The light and flask were enclosed in a metal shield and the reaction was illuminated and stirred for 2 hours. The heat from the light bulb was sufficient to cause the carbon tetrachloride to reflux. The hot mixture was filtered, after which the product precipitated from the filtrate. The

[2] Microanalyses are due to Mr. Elmer F. Shelberg and staff of the Abbott Microanalytical Laboratory.

TABLE III.—COMPARATIVE PHARMACOLOGICAL ACTIVITIES

No.[a]	Relative Activity[b]	No.	Relative Activity
4	3.0	7	1.0
24[c]	2.5	1	1.0
3	2.25	5	1.0
9	2.0	25	0.5
2	1.5	8	0.1

[a] The numbers refer to the compounds in Tables I and II. [b] Activity is rated on a 0–3 scale, in which 3.0 is assigned as the relative activity of our most active compound. In arriving at these relative values, the dose and duration of effect have been given more weight than the actual degree of prolapse. The figures in this table represent approximate values, and are not to be considered definitive. [c] Bretylium iodide.

material was recrystallized once from Skelly B and weighed 63 Gm. (70%), m.p. 107.5–109°. The recorded m.p. is 107–108° (3).

o-Trifluoromethylbenzyl Alcohol.—Reduction of 36 Gm. (0.19 mole) of *o*-trifluoromethylbenzoic acid (4) with lithium aluminum hydride was accomplished in diethyl ether. The yield was 20.5 Gm. (61.4%) of material boiling at 124–126° (57 mm.), n_D^{25} 1.4661.

Anal.—Calcd. for $C_8H_7F_3O$: C, 54.56; H, 4.00. Found: C, 54.08; H, 3.88.

o-Trifluoromethylbenzyl Bromide.—A solution of 20.5 Gm. (0.12 mole) of *o*-trifluoromethylbenzyl alcohol and 4 ml. of pyridine was added, with stirring, dropwise over a period of 3 hours to 12.6 Gm. of phosphorus tribromide at −5 to −10°. After addition was complete, the reaction mixture was slowly allowed to come to room temperature overnight. It was poured onto ice. The white solid which formed was collected and washed well with ice water. On warming to room temperature the solid liquified; it was then distilled at 116–118° (60 mm.), n_D^{25} 1.4967. The yield was 21.5 Gm. (77.5%).

Anal.—Calcd. for $C_8H_6BrF_3$: C, 40.20; H, 2.53. Found: C, 40.11; H, 2.41.

Preparation of Substituted N,N-Dimethylbenzylamines.—The compounds listed below were prepared from dimethylamine and the appropriate benzyl halide according to the procedure of Eliel, Ferdinand, and Herrmann (5): 4-chloro-N,N-dimethylbenzylamine (5), b.p. 104–106° (19 mm.), n_D^{25} 1.5172; 2,4-dichloro-N,N-dimethylbenzylamine (5), b.p. 127–129° (20 mm.), n_D^{25} 1.5370; 2-chloro-N,N-dimethylbenzylamine (6), b.p. 103° (19 mm.), n_D^{25} 1.5212; 2-bromo-N,N-dimethylbenzylamine (7), b.p. 112–113° (17 mm.), n_D^{25} 1.5445; 4-bromo-N,N-dimethylbenzylamine (8), b.p. 98–102° (8 mm.), n_D^{26} 1.5401.

1-Bromo-2-dimethylaminomethylnaphthalene.—It was prepared from 1-bromo-2-bromomethylnaphthalene in the manner described above, b.p. 195–200° (2.5 mm.), n_D^{26} 1.6202.

Anal.—Calcd. for $C_{13}H_{14}BrN$: C, 59.10; H, 5.34; N, 5.30. Found: C, 59.04; H, 5.42; N, 5.15.

N,N-Dimethylfurfurylamine.—This compound was prepared by the Leuckart reaction as previously described (9), b.p. 142–144°, n_D^{25} 1.4592.

N,N-Dimethyl-*o*-methoxybenzylamine (10)—This compound was prepared from *o*-methoxy-N-methylbenzylamine[3] by methylation with formaldehyde and formic acid (11), b.p. 99–101° (10 mm.), n_D^{25} 1.5147.

1,2,2,4,6-Pentamethylpiperidine.—Methylation of 28 Gm. (0.2 mole) of 2,2,4,6-tetramethylpiperidine (Shell Development Co., Emeryville, Calif.) was accomplished with formaldehyde and formic acid (11). The yield of colorless liquid was 20.3 Gm. (65.5%), b.p. 60–61° (12 mm.), n_D^{25} 1.4508.

Anal.—Calcd. for $C_{10}H_{21}N$: C, 77.35; H, 13.63. Found: C, 77.29; H, 14.06.

A sulfate salt was prepared and recrystallized from a mixture of acetone and 2-propanol. It melted at 147–149°.

Anal.—Calcd. for $C_{10}H_{21}N \cdot H_2SO_4$: C, 47.41; H, 9.15; N, 5.53. Found: C, 47.54; H, 8.59; N, 5.52.

Preparation of the Quaternary Salts.—A solution of 0.1 mole of the amine and 0.1 mole of the halide[4] in 50 to 100 ml. of methyl ethyl ketone was allowed to stand at room temperature. The product precipitated after a few hours to several days. The less reactive combinations could be obtained more quickly by refluxing the reaction mixture. If the product precipitated as an oil, it was scratched and chilled until a solid formed. To purify the compounds, they were recrystallized from a suitable solvent such as acetone, methyl ethyl ketone, methanol, ethanol, or 2-propanol. These substances are described in Tables I and II.

(*o*-Chlorobenzyl)(vinyloxyethyl)dimethylammonium Iodide (Table I, No. 3).—A solution of 5.8 Gm. (0.05 mole) of (vinyloxyethyl)dimethylamine (Peninsular Chemresearch, Inc.) and 16 Gm. (0.1 mole) of *o*-chlorobenzyl chloride in 50 ml. of acetone was allowed to stand at room temperature for 3 days and then refluxed for 6 hours. To the solution was added a solution of 15 Gm. (0.1 mole) of sodium iodide in 100 ml. of acetone. After standing overnight at room temperature, the sodium chloride was collected, and the filtrate was concentrated and chilled to obtain 8.8 Gm. (48%) of white solid, m.p. 127–128.5°. One recrystallization from ethanol-acetone raised the melting point to 128.5–129°.

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[3] Prepared in this laboratory by Dr. W. B. Martin.
[4] If the halide was available in large quantities, such as methyl iodide and ethyl iodide, a two- to fourfold excess was used.

Paper Chromatography of Alkaloidal Extracts of *Lobelia* Species

By M. G. CHAUBAL†, R. M. BAXTER, and G. C. WALKER

Total alkaloidal extracts from four closely related *Lobelia* species, *L. cardinalis* L., *L. syphilitica* L., *L. puberula* Michaux, and *L. elongata* Small, and ten crosses and hybrids involving *L. cardinalis* and *L. syphilitica,* were examined using two-phase descending paper chromatography with formamide-ammonium formate-formic acid as the stationary phase and chloroform-benzene as the mobile phase. The chromatogram of *L. elongata* showed the presence of lobinaline, suggesting its closer relationship with *L. cardinalis.* The chromatograms of *L. cardinalis, L. syphilitica,* and *L. puberula* were different from each other, suggesting the elaboration of different alkaloids by each of these related species. Lobinaline was present in the first species and absent in the latter two species. The *L. cardinalis* × *L. syphilitica* hybrids gave chromatograms suggesting the elaboration of alkaloids characteristic of both the parent plants.

As a result of extensive chemical investigations on various plant species, especially those of economic importance, as, for example, vegetable drugs, the occurrence of "chemical races," "physiological races," or "chemical varieties" has been suggested (1, 2). These plants have similar phenotype but different genotype, and as such are identical in external characters but differ in their chemical composition. Study of chemical composition in relationship to the taxonomy of plants and the development of "chemical systematics" or "biochemical systematics" has interested many workers in recent years (3–9). Systematic chemical studies of genera and families have led to the discovery of new and sometimes unexpected structural types. The observation that certain compounds sometimes do not occur in groups of plants where their presence for botanical reasons could be expected, or that these compounds are replaced, has stimulated study of the deeper causes of such anomalies and led to the deeper understanding of the mechanisms of evolution and the later stages of biosynthetic processes.

The genus *Lobelia* has been of interest from the above point of view. *L. inflata* L. contains lobeline as the major alkaloid while *L. nicotianaefolia* Heyne contains similar alkaloids, lobelanidine being the major alkaloid (10). Bowden (11) has correlated taxonomic, ecological, and cytogenic data on 21 species of *Lobelia* L. section *Lobelia* and has observed the relationship between these species. The alkaloidal extracts from four species of *Lobelia: L. cardinalis* L., *L. syphilitica* L., *L. elongata* Small, and *L. puberula* Michaux, and ten crosses and hybrids involving *cardinalis* and *syphilitica*, have been examined by the technique of descending paper chromatography in this work.

EXPERIMENTAL

Plant Materials.—Total alkaloidal extracts of the following four species of *Lobelia* and ten hybrids were obtained.[1] The method of preparation of the extracts was as outlined by Manske (14). The quantity of dried plant material extracted and the total amount of extractive are indicated in brackets following each species or cross (Gm.). X-515: diploid, *cv.* "Illumination" × *L. cardinalis* subsp. *graminea* var. *pseudosplendens* (N. Mex.) (933-3.90); X-517: *cv.* "Queen Victoria" × *L. cardinalis* subsp. *cardinalis* (584-2.55); X-518: *cv.* "Queen Victoria" × *L. cardinalis* subsp. *cardinalis* (677-2.50); X-537: tetraploid, from a double hybrid cross (two F_1's) involving two accessions of *L. cardinalis* subsp. *cardinalis* and two accessions of *L. syphilitica* var. *syphilitica* (545-1.52); X-520: *L. cardinalis* subsp. *cardinalis* × *L. cardinalis* subsp. *graminea* var. *pseudosplendens* (N. Mex.); X-552: *L. cardinalis* subsp. *graminea* var. *graminea* (Mexico) × *L. cardinalis* subsp. *graminea* var. *graminea* (Mexico); X-628: *L. cardinalis* subsp. *graminea* var. *graminea* (Honduras) × *L. cardinalis* subsp. *graminea* var. *graminea* (Mexico) (X-520, X-552, and X-628 were mixed) (256 + 281 + 125-3.24); X-917-S: selfed, complex tetraploid hybrid involving *cardinalis* and *syphilitica* tetraploid: X-918: same as X-917-S, but two seedlings of the parent hybrid were intercrossed to give X-918, tetraploid X-917-S and X-918 were mixed (343-1.28); X-897: tetraploid, complex parentage involving *L. cardinalis* and *L. syphilitica*, had some of the same parentage as X-537 (523-1.80); X-749: tetraploid, involving *L. cardinalis* and *L. syphilitica;* X-909: tetraploid, involving *L. cardinalis* and *L. syphilitica* (X-749 and X-909 were mixed) (179 + 390-1.35); L.C.C.

Received October 26, 1961, from the Faculty of Pharmacy, University of Toronto, Toronto 2, Ontario, Canada.
Accepted for publication January 22, 1962.
This research was supported in part by a grant from the Canadian Foundation for the Advancement of Pharmacy and the National Research Council of Canada, and this support is gratefully acknowledged.
The authors are indebted to Dr. R. H. F. Manske for supplying the alkaloidal extracts, to Dr. W. M. Bowden for supplying much of the plant material, and to Dr. S. I. Kandel for his interest and cooperation during the progress of the investigation.
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[1] The materials were supplied by Dr. R. H. F. Manske, Dominion Rubber Co., Guelph, Ontario, and Dr. W. M. Bowden, Botany and Plant Pathology Laboratory, Department of Agriculture, Ottawa, Ontario.

403-407 (681-2.83); *L. cardinalis* var. *cardinalis* 5-6-B-S7: *L. elongata* Small, grown from seeds collected at Edenton, N. C. (591-2.42); 17-21-S1-3: diploid, mostly *cardinalis* genes but may be a hybrid, from Erfurt, Germany (681-1.08); *L. puberula* Michx.: composite sample of seedlings grown from seeds collected in North and South Carolina and Florida (196-0.35); *L. cardinalis* var. *cardinalis*; *L. syphilitica* var. *syphilitica*.

Chromatographic Procedure.—Preliminary separations showed that Whatman No. 3 chromatographic paper gave better separations, more constant R_f values, and little or no streaking.

The following two-phase system developed by Steinegger and Ochsner (12) was selected following preliminary screening. Stationary phase: 9 ml. of formamide (laboratory reagent) was dissolved in 20 ml. of acetone (distilled at 56–57° and dried over anhydrous sodium sulfate). The solution was saturated with ammonium formate (0.9 Gm. laboratory grade) and the supernatant filtered through dry filter paper. One milliliter of concentrated formic acid (98–99%) was added to the filtrate. Mobile phase: 9 parts of chloroform (analytical) was mixed with 1 part of benzene (analytical).

Whatman No. 3 paper strips, 19 cm. wide and 57 cm. long, were impregnated with the stationary phase by passing the strips through the solution in a flat dish from about 2.5 cm. above the starting line and down to the lower edge. Excess solution was blotted between filter paper sheets and the paper hung in air to remove all acetone. Six spots were applied to the paper in methanol (synthetic) solution. Approximately 400 mcg. of the total alkaloidal extract and 100 mcg. of the pure alkaloid were applied. Each strip carried one spot of lobinaline which served as a control spot. The spotted paper was then allowed to equilibrate with the vapor of the mobile phase in Pyrex glass chromatographic jars for 12 to 15 hours. The chromatogram was developed after this equilibration period until the mobile phase had run approximately 45 cm. from the starting line. The paper strips were then taken out of the jars and dried in air for one-half hour. Formamide was removed from the strips by keeping them in a hot-air oven at 90° for 2 hours. The color of the alkaloidal spots was developed by spraying with modified Dragendorff's reagent (13). The orange-red alkaloidal spots were marked off on the paper with a pencil and the R_f values of

Table I.—R_f Values of the Dragendorff-Positive Spots from the Extracts of Four *Lobelia* Species

L. cardinalis	*L. puberula*	*L. elongata*	*L. syphilitica*
0.00	0.00	0.00	0.00
0 48[a]	0.06	0.03	0.025
0.80	0.1	0.041	0.15[b]
	0.2	0.06	0.25
	0.3	0.46[a]	0.30[b]
	0.52	0.80	0.40
			0.55
			0.75

[a] Rf values for lobinaline. [b] Rf values for lophilacrine and lopheline (12, 13).

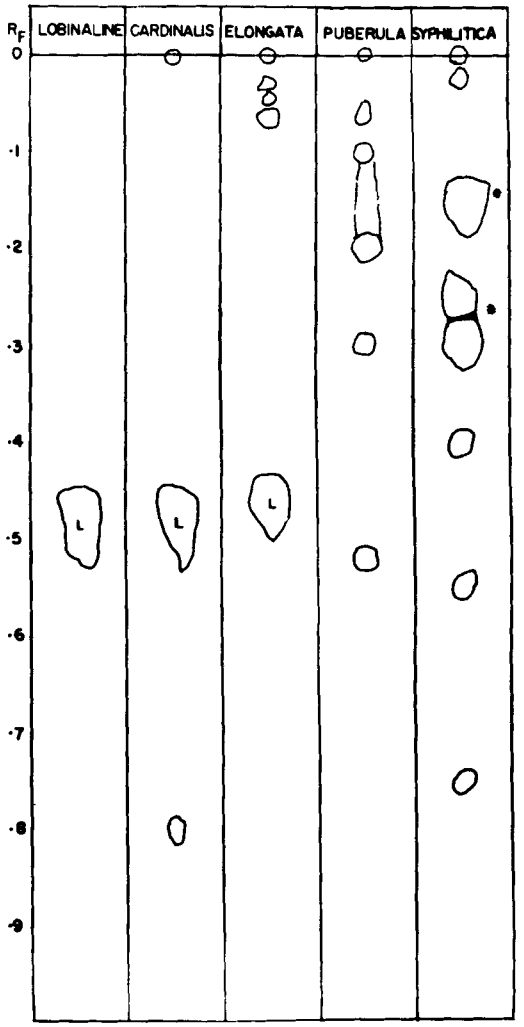


Fig. 1.—Chromatograms of total alkaloidal extracts of four *Lobelia* species. L, lobinaline; *, lopheline and lophilacrine.

Table II.—R_f Values of the Dragendorff-Positive Spots from the Extracts of the *Lobelia* Hybrids

X-515	X-517	X-518	X-537	X-520 X-552 X-628	X-897	X-749 X-909	L.C.C. 403–407	X-917 S X-918	12-21-S1-3
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.06	0.07	0.04	0.06	0.02	0.025	0.025	0.027	0.06	0.06
0.20	0.16	0.07	0.19[b]	0.06	0.08	0.07	0.07	0.15[b]	0.15[b]
0.40	0.3	0.2	0.3[b]	0.18	0.16[b]	0.18[b]	0.5[a]	0.28[b]	0.3[b]
0.52[a]	0.52[a]	0.48[a]	0.5[a]	0.50[a]	0.24[b]	0.3[b]	0.82	0.53[a]	0.54[a]
0.87	0.90	0.87[a]	0.9	0.8	0.49[a]	0.54[a]		0.89	0.72
					0.75	0.87			

[a] Rf values for lobinaline. [b] Rf values for lophilacrine and lopheline (12, 13).

each noted. (See Tables I and II; Figs. 1, 2, and 3.) The R_f values given are only a guide and are average values of about 10 determinations. The variables governing R_f values are numerous and, for this reason, the locator spot of a solution of pure crystalline lobinaline was applied on each strip.

RESULTS

The chromatograms and R_f values (Figs. 1, 2, and 3 and Tables I and II) were compared with each other on the basis of the presence or absence of lobinaline (14). Other alkaloids in these species have not been isolated or characterized to any extent. *L. cardinalis* L. gave three spots, and that corresponding to lobinaline at R_f 0.46 to 0.53 was the most prominent (Fig. 1). *L. syphilitica* L. gave eight spots, and the two prominent spots at R_f 0.16 and 0.3 have been attributed to the presence of two liquid alkaloids, lopheline and lophilacrine, respectively (15). This species did not appear to contain lobinaline. *L. puberula* Michaux gave six small spots and the chromatograms would indicate an absence of lobinaline in this species also. The chromatograms appeared to be different from those of the extracts from the other three species (Fig. 1). *L. elongata* gave six spots. The prominent spot at R_f 0.46 was eluted out and rechromatographed after mixing with pure lobinaline. Only one spot was obtained, thus indicating the presence of lobinaline in this species.

Chromatograms of the extracts from crosses involving *L. cardinalis* varieties alone, X-515, X-517, X-518, X-520 + X-552 + X-628, and L.C.C. 405-407 (Fig. 2) showed the presence of lobinaline. In these cases there were a greater number of alkaloids (5 to 6 spots) than the three elaborated by *L. cardinalis* L. The hydrids involving *L. cardinalis* L. and *L. syphilitica*, X-537, X-917-S + X-918, X-897, X-909 + X-749 (Fig. 3), gave chromatograms showing spots due to lobinaline and also due to lopheline and lophilacrine. The extract 12-21-S1-3 gave spots corresponding to lobinaline, lophilacrine, and lopheline (Fig. 3).

DISCUSSION

According to Bowden (11), *L. elongata* Small appears to have originated by the crossing of a *puberula*-like ancestor and some other closely related species. The presence of lobinaline and the chromatograms in general with this species would appear to indicate a closer relationship to *cardinalis* than to *puberula*. Bowden has also observed that although strong morphological differences distinguish *L. cardinalis* from *L. syphilitica*, cytogenetically these two species are certainly related, *L. cardinalis* having been derived by some mutational process from a *syphilitica*-like ancestral species, the

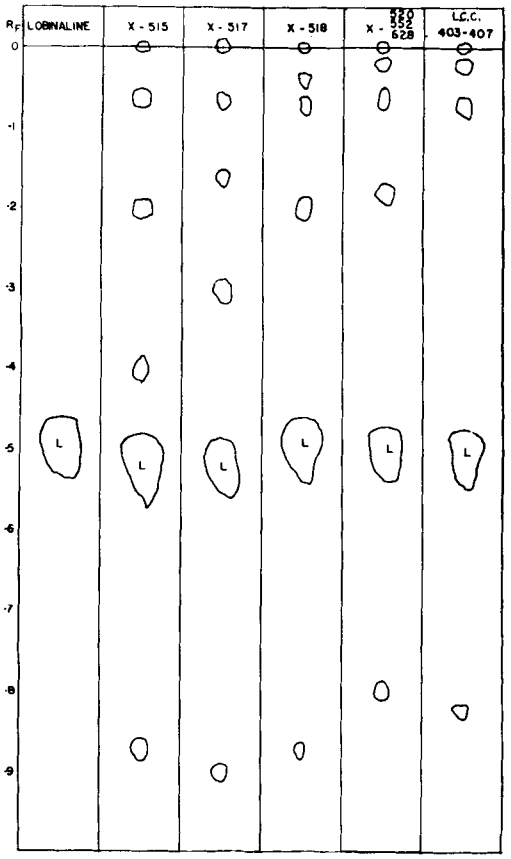


Fig. 2.—Chromatograms of total alkaloidal extracts of crosses involving *L. cardinalis* L. L, lobinaline.

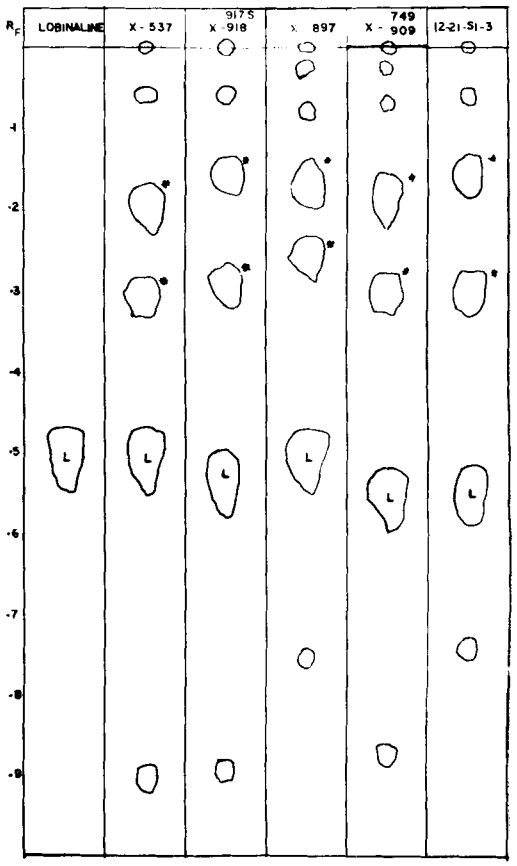


Fig. 3.—Chromatograms of total alkaloidal extracts of *L. cardinalis* and *L. syphilitica* crosses. L, lobinaline; *, lopheline and lophilacrine.

mutational changes involving changes in chromosome structure. The chromatograms of these two species showing the occurrence of lobinaline in the first species and its absence in the latter would suggest that these mutational changes have also resulted in changes in alkaloidal composition and in structural type in these two species. *L. syphilitica* and *L. puberula* have been shown to be cytogenetically related by Bowden. The chromatograms of these two species do not seem to show any close relationship. The *cardinalis* × *syphilitica* hybrids produce alkaloids from both the parent plants. These observations on *L. syphilitica* and the *cardinalis* × *syphilitica* hybrids support and extend the observations of Steinegger, *et al.* The plant 12-21-S1-3 (Fig. 3) was observed to be a diploid plant with mostly *cardinalis* genes but which might have been a hybrid. The chromatograms having spots due to lobinaline and lopheline and lophilacrine indicate that the sample was a hybrid involving *cardinalis* and *syphilitica*.

The chromatograms of *L. cardinalis* L. (Fig. 1 and Table I) show three spots: R_f 0.00, 0.48, and 0.80. Kaczmarek and Steinegger (16) obtained only two spots, R_f 0.45 and 0.86, with fresh extracts of the species. However these same authors observed an increased number of spots (5–8) with a 6-year-old petroleum ether extract.

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Bisulfite Reduction of *p*-Nitrobenzyl Alcohol

By LOUIS C. SCHROETER and TAKERU HIGUCHI

Bisulfite reduction of *p*-nitrobenzyl alcohol appears to follow a Piria mechanism leading to formation of α-hydroxy-6-sulfoamino-*m*-toluenesulfonic acid. Stoichiometry of the reaction involves one mole of nitro compound and three moles of bisulfite; pH profile of the initial rate of the stoichiometric reaction has been studied. Preliminary kinetic studies indicate an apparent heat of reaction of about 22 Kcal. mole^{-1}. This complicated sequential reduction reaction appears to involve two mechanistic pathways: a rate-determining initial S_N1 reaction dominates at pH 4, while at pH 7 the initial rate-determining reaction appears to possess typical S_N2 characteristics.

BISULFITE REACTIVITY toward the *p*-nitrobenzyl alcohol moiety of chloramphenicol was demonstrated during an investigation of the stability of the antibiotic in the presence of the thio compound (1). Complexity of the antibiotic molecule made evaluation of the kinetic results rather difficult and, for this reason, *p*-nitrobenzyl alcohol was employed as a model compound for the antibiotic so that certain mechanistic details of the reaction could be evaluated. Spectrophotometric and chromatographic evidence obtained during the kinetic study of both the antibiotic and the model compound indicated involvement of nitro reduction with formation of a very polar product; this served to differentiate the reaction from that which occurs between nitrobenzyl halides and sulfite (2). Nitro reduction plays only a minor role under reaction conditions which lead to near-quantitative yields of the nitrobenzyl sulfonic acid from the corresponding nitrobenzyl halides.

The reaction of either *p*-nitrotoluene or *p*-nitrobenzoic acid with sulfite in dilute aqueous solution under conditions similar to those employed with the *p*-nitrobenzyl alcohol leads to formation of both sulfoamino and sulfoaminosulfonic acid compounds (3, 4). This reduction-sulfonation reaction is known as the Piria reaction (3). The mechanism of the Piria reaction for *p*-nitrotoluene analogs of *p*-nitrobenzyl alcohol and other aromatic nitro compounds has been treated

Received October 14, 1961, from The Pharmacy Research Section, Product Research and Development, The Upjohn Co., Kalamazoo, Mich., and The School of Pharmacy, The University of Wisconsin, Madison.
Accepted for publication November 20, 1961.
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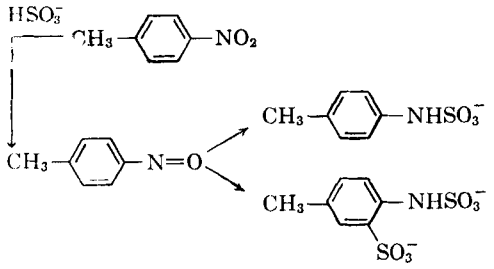
Bisulfite reduction of *p*-nitrobenzyl alcohol appears to follow a Piria mechanism leading to formation of α-hydroxy-6-sulfoamino-*m*-toluenesulfonic acid. Stoichiometry of the reaction involves one mole of nitro compound and three moles of bisulfite; pH profile of the initial rate of the stoichiometric reaction has been studied. Preliminary kinetic studies indicate an apparent heat of reaction of about 22 Kcal. mole^{-1}. This complicated sequential reduction reaction appears to involve two mechanistic pathways: a rate-determining initial S_N1 reaction dominates at pH 4, while at pH 7 the initial rate-determining reaction appears to possess typical S_N2 characteristics.

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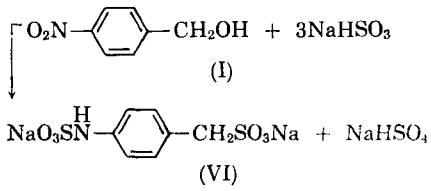
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in detail (3–6), and the following pathway has been suggested (6) for the reaction of *p*-nitrotoluene with sulfite in aqueous systems

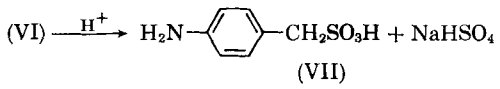


Hunter and Sprung (3) reported that the main product (72%) of the reaction was *p*-tolylsulfamic acid, while under similar conditions with *p*-nitrobenzoic acid as the reactant, the nuclear sulfonated product, 3-sulfo-4-sulfoaminobenzoic acid, was the main product (61%). The analogous mechanistic pathways for the *p*-nitrobenzyl alcohol-bisulfite reaction would be as follows

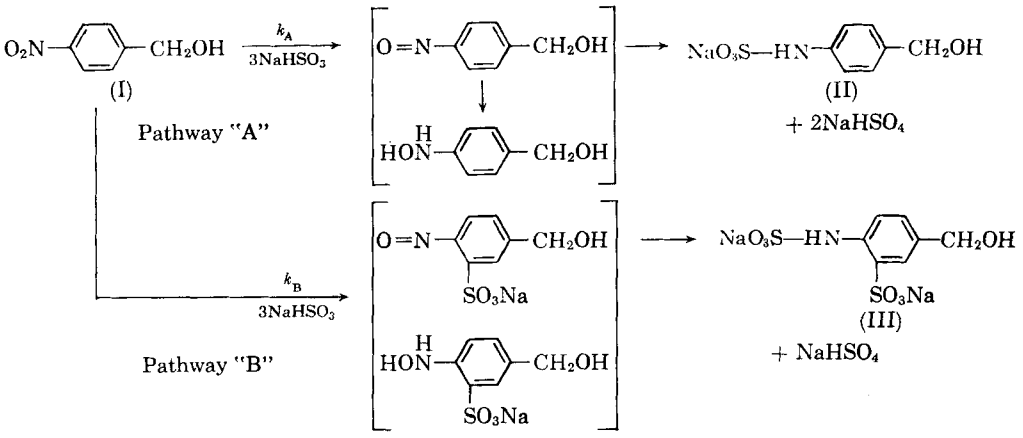
Isolation of product is of paramount significance in considering mechanisms because the following postulated reaction also consumes three moles of sulfite and produces a sulfoamino-sulfonic acid product



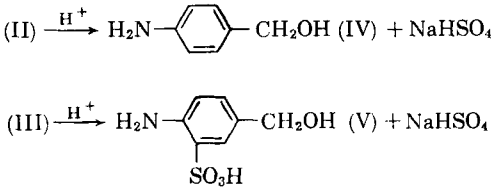
Acid Hydrolysis of Product:



Validity of this postulated mechanism could be tested simply by subjecting the product to acid hydrolysis; this would yield the slightly soluble zwitterionic *p*-aminobenzylsulfonic acid (VII). The highly polar nature of the products proposed



Acid Hydrolysis of Products:



Applicability of the above mechanism and determination of the main pathway (A or B) requires evaluation of several factors: (*a*) stoichiometry of reaction, i.e., total loss of bisulfite with respect to nitro compound; (*b*) molar yield of inorganic sulfate obtained by acid hydrolysis of reaction mixture; (*c*) product isolation.

in the above schemes makes isolation from contaminating inorganic sulfate very difficult. Repeated ethanolic extractions have been used with some success for the isolation of products from Piria reactions (3, 4). Although this technique is rather cumbersome, it serves to isolate all possible products under mild conditions. The procedure described by Nomine (7) for the isolation of a steroid sulfaminate offers a unique, rapid method in which the product is isolated as a water-insoluble quaternary ammonium salt. This latter procedure is generally applicable to the isolation of all postulated products from the reaction of sulfite with *p*-nitrobenzyl alcohol.

EXPERIMENTAL

Stoichiometry and Kinetic Studies

Solutions of *p*-nitrobenzyl alcohol and sodium bisulfite were prepared separately in various 0.3 *M* buffers. The nitro compound was dissolved in 150 ml. of buffer and the bisulfite in 50 ml. of nitrogen-flushed buffer. Ionic strength of the final combined solution (200 ml.) was maintained constant at 1.0 by addition of appropriate amounts of sodium chloride to the bisulfite solution. Solutions were equilibrated separately at the reaction temperature, then rapidly and simultaneously transferred to a reaction vessel immersed in a thermostat; this marked "zero time" for the reaction. Reaction solution was stirred at a fixed rate under nitrogen atmosphere during course of the reaction. Thermostat temperature was maintained ±0.1°. The pH of the reaction mixture was determined at the temperature of the study with a Beckman Zeromatic pH meter standardized against known buffers maintained at the same temperature. Sampling was performed by periodically withdrawing 5-ml. aliquots from the reaction mixture and quenching in an ice-water bath. Bisulfite content was determined by iodometric titration. Concentration of *p*-nitrobenzyl alcohol and of its reduction product was determined by ultraviolet spectrophotometry. Appropriate dilutions of the aliquots were made with pH 6.50, 0.01 *M* phosphate buffer; absorption was rapidly recorded from 350 to 220 mμ using a Cary model 11 recording spectrophotometer. Resultant spectra were evaluated by two component analysis using 235 and 277 mμ maxima.

Acid Hydrolysis and Sulfate Determination

Reduction Reaction.—Six millimoles (0.919 Gm.) of *p*-nitrobenzyl alcohol (Eastman WL recrystallized twice from water, m.p. 93°) were dissolved in 150 ml. of nitrogen-flushed water maintained at 55°. Sulfite solution was prepared to contain 12 mmoles (1.249 Gm.) of sodium bisulfite A.R. and 12 mmoles (1.543 Gm.) of sodium sulfite A.R. in 50 ml. of water at 55°. The solutions were simultaneously introduced into a reaction vessel fitted with a dropping funnel, nitrogen flushing tube, and a gas exit tube. The reaction was maintained at pH 6.5 ± 0.3 by appropriate additions of acid or base. The solution was thermostatted at 55° for 7 hours while water-saturated nitrogen bubbled through to mix the solution and to prevent autoxidation of sulfite.

Hydrolysis Reaction.—Subsequent to the reduction reaction, 30 ml. of concentrated hydrochloric acid was slowly added to the reaction solution through the dropping funnel. Flow of nitrogen through the solution was increased to expel sulfur dioxide; the exit tube was then connected to a sodium hydroxide absorption train. The acid hydrolysis reaction was maintained at 55° for 48 hours; nitrogen was constantly bubbled through the solution to expel sulfurous acid.

Sulfate Determination.—Barium chloride solution was added dropwise to the acidified hydrolysis reaction solution until no further precipitation of barium sulfate occurred. The collected precipitate was digested, washed, ignited, and weighed in the customary manner. The weight of barium sulfate from similarly treated blank containing no *p*-nitrobenzyl alcohol was subtracted from sample value to give net inorganic sulfate formed on hydrolysis: 12.32 mmoles $BaSO_4$ from sample minus 0.23 mmoles $BaSO_4$ from blank equals 12.09 mmoles $BaSO_4$ from sample hydrolysis. Ratio of sulfate obtained on hydrolysis to react nitro compound (PNBA): 12.09 sulfate/6.0 PNBA = 2.015 moles sulfate per mole PNBA.

Isolation of Acid Hydrolysis Product

Reduction reaction was carried out as described above. The following variations in hydrolysis conditions were employed

Added HCl, ml.	Hydrolysis—Time, hr.	Hydrolysis—Temp., ° C.	Product Analyses—Carbon, %	Product Analyses—Hydrogen, %
30	48	55	47.92	4.67
20	48	55	46.38	4.50
10	48	55	42.67	3.97
10	12	55	37.53	3.77
10	12	35	29.84	3.20
10	6	25	25.98	3.34

After cooling, the solutions were extracted with five 50-ml. portions of diethyl ether. The combined ethereal extracts were evaporated to dryness and weighed. Less than 10 mg. residue was obtained. The pH of the aqueous acid solution was adjusted to 2.5 by the addition of sodium carbonate. The yellow precipitate which formed was filtered from solution after 24 hours and dried under vacuum. Combustion analyses of products are tabulated above. Filtrate pH was adjusted to 9 with carbonate. The solution was extracted with five 50-ml. portions of chloroform. Chloroformic solutions evaporated to dryness yielded less than 10 mg. residue. Infrared spectra of products were compared with the known spectrum of *p*-aminobenzylsulfonic acid prepared as described below.

Product Isolation.—*A. Isolation of Sodium α-Hydroxy-6-sulfoamino-m-toluene Sulfonate (III) from a pH 6.5 Reduction Reaction.*—The reduction reaction between a fourfold molar excess of bisulfite with *p*-nitrobenzyl alcohol as described above was repeated at 55° with the pH maintained at 6.5.

One hundred milliliters of a reaction solution containing 3 mmoles of product, as shown by spectrophotometric analysis, was allowed to evaporate spontaneously to dryness at ambient temperatures. Care was taken in all subsequent operations to avoid temperatures higher than 25° so that product decomposition would be minimized. The dry residue was extracted with three 25-ml. portions of diisopropyl ether. The combined ethereal extracts, evaporated to dryness, yielded less than 5 mg. residue. The dried reaction residue was then extracted with four 50-ml. fractions of 65% ethanol. The combined ethanolic solutions were evaporated to dryness. The resultant residue was extracted with 200 ml. of 75% ethanol; the procedure was repeated using 85 and 95% ethanol. Residue from the 95% ethanol extraction was dissolved in 5 ml. of water and filtered. Residue from the aqueous filtrate was recrystallized four times from water-ethanol. Yield: 476 mg. (48.5%).

Anal.—Calcd. for $C_7H_7NNa_2O_7S_2$ (III): C, 25.69; H, 2.16; N, 4.28; S, 19.59. Found: C, 25.93; H, 4.08; N, 4.06; S, 16.97. Ultraviolet spectrum in water: ε 235.5 mμ = 10,850; ε 280 mμ = 1600. Infrared spectrum shows the following bands: –OH/–NH: 3550, 3425, 3300 cm.$^{-1}$; $-SO_3-$: 1300, 1250, 1230, 1170 cm.$^{-1}$; C–O: 1040.

B. Isolation of Sodium α-Hydroxy-6-sulfoamino-m-toluene Sulfonate (III) from a pH 4 Reduction Reaction.—The isolation was carried out exactly as described for the pH 6.5 product. Yield: 394 mg. (40.2%).

Anal.—Calcd. for $C_7H_7NNa_2O_7S_2$ (III): C, 25.69; H, 2.16; N, 4.28. Found: C, 25.15; H, 3.07; N, 4.26. The infrared spectrum was identical with that obtained from the pH 6.5 product.

C. Isolation of Potassium α-Hydroxy-6-sulfoamino-m-toluene Sulfonate (III) from a pH 6.5 Reduction Reaction.—An aliquot of a pH 6.5 reduction reaction was diluted to 100 ml., cooled to 25°, and extracted with six 50-ml. fractions of diisopropyl ether. The combined ethereal extracts evaporated to dryness yielded less than 10 mg. residue. A 150-ml. quantity of 5% benzethonium chloride solution was added slowly with stirring to the aqueous phase. The solution was allowed to stand 24 hours at 5° before pouring off the supernatant aqueous solution from the oily residue. The oily residue was dissolved in 200 ml. chloroform which was then washed with three 75-ml. portions of water and passed through a 2 × 20 cm. column containing anhydrous sodium sulfate. Eluate volume was adjusted to 200 ml. with chloroform rinsings from the column: eluate was evaporated to dryness under nitrogen at 25°. Residue was dissolved in 150 ml. absolute ethanol, and 50 ml. of 1 *M* ethanolic potassium acetate was added slowly with stirring. After 24 hours standing at 5°, the yellow precipitate was filtered from solution. Product recrystallized twice from ethanol-water.

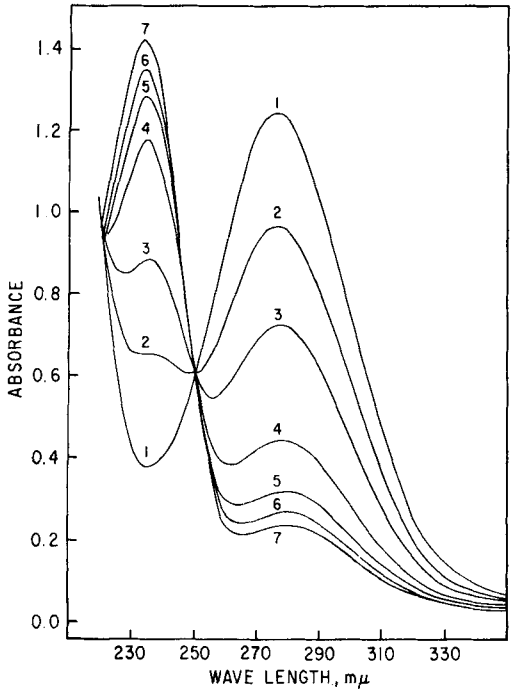


Fig. 1.—Ultraviolet spectra showing reduction of nitro compound (277 mμ) to sulfoamino product (235 mμ) in presence of excess bisulfite at 55°. Initial solution composition: 0.03 *M* *p*-nitrobenzyl alcohol and 0.12 *M* total sulfite in pH 6.50, 0.3 *M* buffer. Final ionic strength adjusted to 1.0. Figure redrawn from original spectrogram.

Anal.—Calcd. for $C_7H_7K_2NO_7S_2$: C, 23.39; H, 1.96; N, 3.90; S, 17.85. Found: C, 22.65; H, 1.91; N, 3.79; S, 16.82.

4-Aminobenzylsulfonic Acid (VII) Synthesis

Five mmoles (1.20 Gm.) of *p*-nitrobenzyl sulfonic acid, sodium salt synthesized by the procedure of Clutterbuck and Cohen (3), was dissolved in 50 ml. of water containing 1.4 Gm. finely powdered iron. The solution was acidified with acetic acid and warmed (70–80°) on a steam bath for 2 hours. After cooling, the solution was filtered. The filtrate was acidified with dilute hydrochloric acid and allowed to stand 24 hours at 5°, yielding light yellow crystals. The product was recrystallized three times from dilute hydrochloric acid. Yield: 465 mg. (49.6%).

Anal.—Calcd. for $C_7H_9NO_3S$ (VII): C, 44.90; H, 4.85; N, 7.48; S, 17.13. Found: C, 45.08; H, 4.91; N, 7.38; S, 16.94. The pKa determined in water was 4.56. Ultraviolet spectrum in water showed the following absorption maxima: ε 239.5 mμ = 4100; ε 283 mμ = 232; ε 325 mμ = 65. Infrared spectrum showed the following bands: –NH: 3180, 3040 cm.$^{-1}$; C=C/–NH deformation: 1628, 1582, 1540 cm.$^{-1}$; $-SO_3^-$/C–N: 1222, 1210, 1168, 1130, 1033, 1030 cm.$^{-1}$.

RESULTS AND DISCUSSION

Stoichiometry of the reaction was followed by iodometric titration of available sulfite and by two-component analysis of ultraviolet spectral curves such as Fig. 1. Rapid ultraviolet spectrophotometry of reduction reactions showed the presence of only two components and a single isosbestic point which suggested that the slow, rate-determining step in the sequential reaction was followed by more

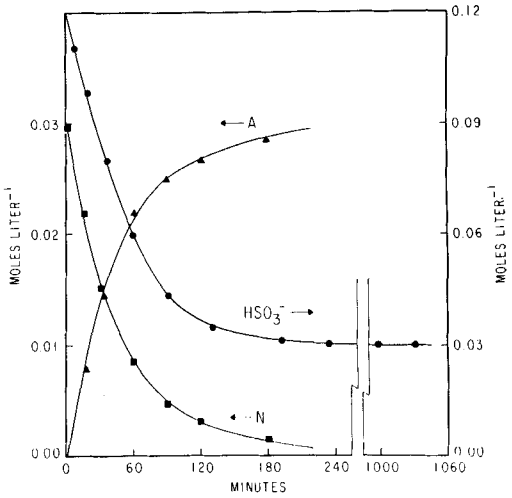


Fig. 2.—Reduction of nitro compound (*N*) with formation of product (*A*) and loss of bisulfite at 55° from solution containing excess bisulfite: 0.03 *M* *p*-nitrobenzyl alcohol and 0.12 *M* total sulfite in pH 6.50, 0.3 *M* buffer. Final ionic strength adjusted to 1.0. Molar concentration of *p*-nitrobenzyl alcohol (■) and its reduction product (▲) indicated by ordinate values to the left of the figure, while total molar concentration of sulfite (●) indicated by right ordinate values.

rapid steps so that the concentration of intermediates was always quite low. Excess bisulfite leads to total conversion of nitro compound with consumption of three moles of bisulfite for each mole of product formed, as shown in Fig. 2. Prolonged reaction times do not lead to further loss of bisulfite, thus establishing the ratio of three moles of bisulfite to one mole nitro compound; this was further verified by repeating studies at higher temperatures for prolonged periods, as shown in Table I. Equimolar concentrations of nitro compound and bisulfite lead to total consumption of bisulfite with formation of one-third molar concentration of product, as shown in Fig. 3.

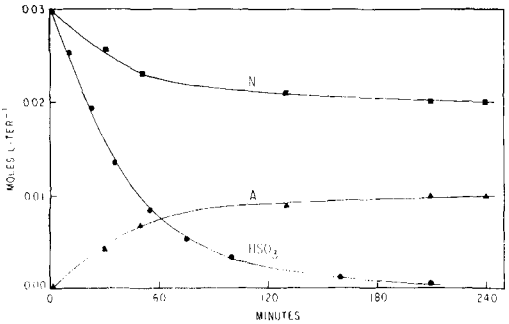


Fig. 3.—Reduction of nitro compound (N) with formation of product (A) and loss of bisulfite at 55° from solution containing excess nitro compound: 0.03 M p-nitrobenzyl alcohol and 0.03 M total sulfite in pH 6.50, 0.3 M buffer. Final ionic strength adjusted to 1.0.

TABLE I.—STOICHIOMETRY OF p-NITROBENZYL ALCOHOL BISULFITE REACTION AT pH 6.50 AND VARIOUS TEMPERATURES[a]

Temperature, °C.	—Bisulfite Concentration (moles L.⁻¹) at— 0 hr.	4 hr.	8 hr.	24 hr.
55	0.120	0.030	0.029	0.030
65	0.120	0.029	0.031	0.030
80	0.120	0.030	0.030	0.029

[a] Solution buffered with 0.30 M phosphate contained 0.03 M p-nitrobenzyl alcohol and 0.12 M sulfite. Ionic strength adjusted to 1.0 by addition of sodium chloride.

Total bisulfite consumption appears to be the same regardless of which mechanism is followed. Acidification of the reduction reaction followed by determination of inorganic sulfates is a powerful tool for establishing which mechanism predominates under given conditions: excess unreacted sulfite is driven off as volatile sulfurous acid, while total sulfate formed during reduction and acid hydrolysis is quantitatively recovered as the insoluble barium salt. Only the sulfoamino group is split under these conditions of acid hydrolysis; nuclear sulfonates are not affected. Isolation of approximately two moles of sulfate upon formation and subsequent hydrolysis of one mole of reaction product strongly supports pathway B of the Piria mechanism; nuclear sulfonation appears to predominate. Isolation of products from acid hydrolyses carried out under varied conditions yields products of variable composition. Under relatively mild conditions (6 hours at 25°), little or no sulfoamino hydrolysis takes place; this is confirmed by both infrared and combustion analysis. More rigorous conditions (30 ml. hydrochloric acid, 55° for 24 hours) lead to product decomposition as evidenced by infrared changes in bonded —NH and —OH. This is not too surprising since aryl sulfamates are known to be alkylating agents at higher temperatures (8). Optimal hydrolysis conditions appear to be at 55° for 48 hours; however, these and other conditions failed to yield infrared evidence for presence of either p-amino-benzylsulfonic acid or p-aminobenzyl alcohol hydrochloride. Reaction solutions made weakly basic, concentrated, and extracted with ether also failed to yield evidence for presence of p-aminobenzyl alcohol.

Product isolation under conditions of acid hydrolysis was complicated by observed tendency of the product to polymerize with heating. Also, strongly acidic conditions introduce the uncertainty factor of secondary reactions leading to artifacts. Isolation of the same product (III) from pH 4 and pH 6.5 reactions by two different procedures in which rigorous conditions were not employed strongly supports mechanism B. It is especially noteworthy that neither isolation procedure is especially selective, i.e., all possible sulfoamino products would be isolated. This is especially significant in considering possible mechanisms since 4-sulfoaminobenzyl alcohol (II) would be isolated under identical conditions if it were present in substantial amounts.

Dependence of the absolute initial rate of loss of bisulfite, $-d(\mathrm{Bi})/dt$, on pH is shown in Fig. 4, in which stoichiometric amounts of reactants (3 bisulfite:1 nitro compound) were reacted at 55°. The initial rate of reaction remains very nearly invariant from pH 3 to 6 exhibiting a rather complex relationship toward hydrogen ion concentration at values above 6. There appears to be a different sensitivity toward singly and doubly charged thio compound: the marked rate increase at pH 7 corresponds to higher initial concentrations of sulfite ion. Actually, considerable concentrations of pyrosulfite, $S_2O_5^{2-}$, ion may be present in concentrated bisulfite solutions (pH 4); however, this species exists in equilibrium with bisulfite ion (9). De-

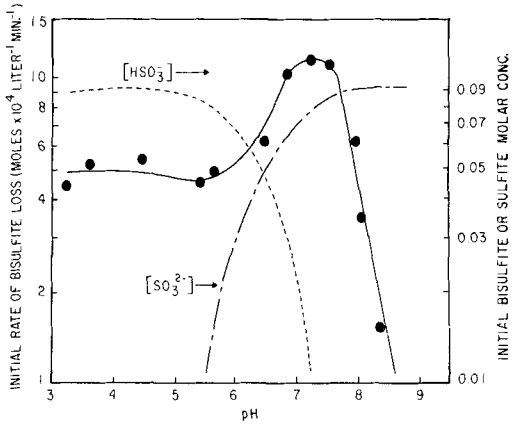


Fig. 4.—Semilog plot of absolute initial rate of bisulfite loss at 55° as a function of pH shown as solid circles joined by solid line. Initial solution composition: 0.03 M p-nitrobenzyl alcohol and 0.09 M total sulfite in 0.3 M buffer; total ionic strength adjusted to 1.0. Initial bisulfite or sulfite ion concentration at 55° in buffers (apparent second ionization constant of sulfurous acid, $pK'_2 = 6.5$) represented by dashed lines.

crease in reaction rate between pH 7.5 and 8 appears dependent on bisulfite ion concentration, but this effect may be more apparent than real inasmuch as this may reflect sensitivity toward increasing hydroxyl ion concentration.

The dependency of the initial rate of nitro loss on initial bisulfite or sulfite concentration is shown in Fig. 5. The initial reaction at pH 4.2 exhibited little dependence on bisulfite ion, and extrapolation of rate values to zero bisulfite concentration suggested that the slow, rate-determining step may involve a S_N1 mechanism. At higher pH values the intercept rate value, [Bi] → 0, decreases, becoming zero at pH values above 7. Plotting intercept rate values, [Bi] → 0, of the initial rates of nitro loss as a function of pH yields Fig. 6 which shows that the initial rate-determining reaction has S_N1 characteristics below about pH 4.6 while S_N2 characteristics predominate above pH 7. The following scheme appears consistent with observed results on the absolute initial rates of nitro loss from *p*-nitrobenzyl alcohol (PNBA) solutions in the presence of bisulfite or sulfite ion

$$\text{PNBA (I)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{(PNBA*)}$$

$$+ SO_3^{2-} \xrightarrow[k_3]{HSO_3^-} \text{Reduction Product (III)}$$

$$\downarrow k_4$$

$$\text{(III)}$$

$$-d(\text{PNBA})/dt = k_4(\text{PNBA})(SO_3^{2-}) + k_3(\text{PNBA*})(HSO_3^-) \quad \text{(Eq. 1)}$$

$$d(\text{PNBA*})/dt = k_1(\text{PNBA}) - k_2(\text{PNBA*}) - k_3(\text{PNBA*})(HSO_3^-) = 0 \quad \text{(Eq. 2)}$$

$$(\text{PNBA*}) = \frac{k_1(\text{PNBA})}{k_2 + k_3(HSO_3^-)} \quad \text{(Eq. 3)}$$

Substituting for (PNBA*) from Eq. 3 into Eq. 1, we have

$$-d(\text{PNBA})/dt = k_4(\text{PNBA})(SO_3^{2-}) + \frac{k_3 k_1(\text{PNBA})(HSO_3^-)}{k_2 + k_3(HSO_3^-)} \quad \text{(Eq. 4)}$$

At pH 4, the initial, rate-determining, reduction reaction may be approximately described by Eq. 5

$$-d(\text{PNBA})/dt = k_1(\text{PNBA})/k_2 \quad \text{(Eq. 5)}$$

which clearly indicates the proposed S_N1 characteristics and is in accord with observed results.

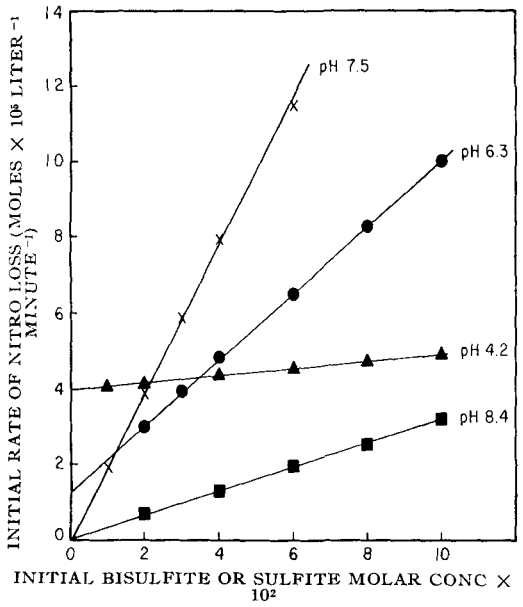


Fig. 5.—Initial rate of nitro loss, $-d(\text{Nitro})/dt$, as a function of initial bisulfite or sulfite ion concentration from buffered (0.3 *M*, μ1.0) solutions at 55°. Solutions initially contained 0.01 *M* *p*-nitrobenzyl alcohol.

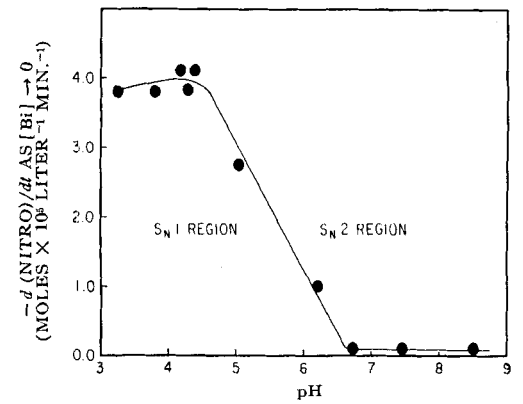


Fig. 6.—Initial rates of nitro loss, $-d(\text{Nitro})/dt$, from bisulfite-*p*-nitrobenzyl alcohol reactions extrapolated to zero bisulfite ion concentration, [Bi] → 0, (*cf.* Fig. 5) plotted as a function of pH. Buffered solutions (0.3 *M*, μ 1.0) initially contained 0.01 *M* *p*-nitrobenzyl alcohol and were thermostatted at 55°.

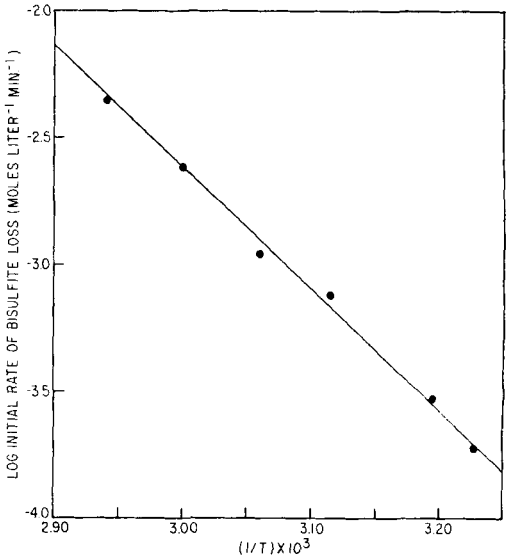


Fig. 7.—Log of initial rate of bisulfite loss from pH 7.0 solutions as a function of reciprocal absolute temperature. Initial solution composition: 0.03 *M* *p*-nitrobenzyl alcohol and 0.09 *M* total sulfite in 0.3 *M* buffer. Final ionic strength adjusted to 1.0.

While at pH 7, the initial, rate-determining, reduction reaction shows dependence on both the nitro and thio species, indicating that Eq. 4 may be simplified to

$$-d(\text{PNBA})/dt = k_4 (\text{PNBA}) (SO_3^{2-}) \quad \text{(Eq. 6)}$$

to describe the S_N2 initial reaction which dominates at higher pH values.

The apparent heat of activation of the initial reaction between stoichiometric amounts of reactants (3 bisulfite:1 nitro compound) determined at pH 7 where S_N2 characteristics dominate gave the value 22 Kcal. mole^{-1} (Fig. 7).

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Thioacetamide II

Substitute for Hydrogen Sulfide in the Quantitative Analysis of Medicinal Chemicals

By M. A. GHAFOOR and C. LEE HUYCK†

Assay procedures utilizing thioacetamide and hydrogen sulfide gas have been compared. The advantages of using thioacetamide are discussed.

THE PURPOSE of this study is to investigate the use of thioacetamide as a substitute for hydrogen sulfide in the analysis of medicinal chemicals. A survey of the literature concerning the use of thioacetamide as a substitute for hydrogen sulfide has been made by Jue and Huyck (1).

Swift and Butler (2) reported that since the rate of acid-catalyzed hydrolysis follows the equation

$$\frac{d\text{TA}}{dt} = -K\,\text{H}^+\,\text{TA}$$

(where TA = thioacetamide), the value of K is 0.21 L. mole^{-1} min.$^{-1}$ at 90° and 0.019 L. mole^{-1} min.$^{-1}$ at 60°. The activation energy is 19.1 Kcal. per mole. These figures mean that in a solution which is 1 M in hydronium ion, thioacetamide would be half hydrolyzed at 100° in 100 seconds, at 60° in 36 minutes, and at 25° in 21 hours. In solutions of pH 10 or more, hydrolysis is quite rapid and yields both thioacetate ions and acetamide.

The relation of hydronium ion concentration to the time of precipitation of arsenic in 0.1 M thioacetamide was studied by Butler and Swift (3). Two kinds of reactions with thioacetamide in sulfide precipitations were proposed: (*a*) hydrolysis controlled reaction in which hydrogen sulfide or hydrosulfide ions are formed as intermediates; (*b*) a direct reaction of metal ions with thioacetamide itself. Thioacetamide is a more rapidly acting reducing agent than hydrogen sulfide since arsenic acid is reduced much faster with thioacetamide than with hydrogen sulfide. The rate of precipitation of arsenic with valence of three by thioacetamide was measured in solutions from pH 1.0 to 3.8 and was found to follow quantitatively the calculated rate of hydrolysis of thioacetamide to hydrogen sulfide.

Molybdenum in titanium alloys was determined by precipitation as sulfide with thioacetamide by McNerney and Wagner (4). Stoner and Finston (5) clearly separated uranium from 200 times its weight of bismuth by precipitating bismuth sulfide from solution. Such a separation is impossible with gaseous hydrogen sulfide without a long digestion period.

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Received January 30, 1962, from the St. Louis College of Pharmacy, St. Louis 10, Mo.
Accepted for publication February 26, 1962.
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Butler, *et al.* (6). If the solubility product is high, precipitation with thioacetamide may be incomplete due to insufficient hydrogen sulfide in the solution.

In volumetric analysis titrations, thioacetamide was used as a reagent for determination of silver in photographic fixing solutions by Bush, *et al.* (7). The solutions were made 0.1 to 1.0 *N* in sodium hydroxide and titrated with 0.01 *N* thioacetamide to an end point determined potentiometrically, using the silver sulfide electrode. This method made it possible to overcome the interference of thiosulfate and halide ions in the usual Volhard titration of silver

Interference of other divalent ions such as Cu^{++}, Cd^{++}, Zn^{++}, Co^{++}, and Ni^{++} was prevented by adding ethylenediaminetetraacetic acid tetrasodium salt.

Using hydrogen sulfide, zinc can be precipitated completely at pH 3.0, or even less, but thioacetamide precipitated only a very little zinc at this pH (8). One remedy is to use a large excess of thioacetamide and perform the precipitation under pressure in a closed vessel. Hydrogen sulfide gas escapes faster than it is generated in the hydrolysis of thioacetamide solutions when they are heated. The solution of thioacetamide should not be boiled during

TABLE I.—ASSAY OF MERBROMIN BY PRECIPITATION WITH HYDROGEN SULFIDE GAS

No.	Weight of Sample, Gm.	Weight of Precipitate, Gm.	% Weight of Mercury
1	0.2611	0.0780	25.76
2	0.2690	0.0785	25.16
3	0.3391	0.1020	25.93
4	0.3201	0.0986	26.57
5	0.2987	0.0891	26.60
6	0.3467	0.1032	25.66
7	0.3303	0.0989	25.82
8	0.3316	0.1001	26.02
9	0.2850	0.0844	25.53
10	0.3110	0.0927	25.70
11	0.3288	0.0954	25.01
12	0.2706	0.0785	25.02
			308.78
		Mean =	25.73

$$\text{S.D.} = \sqrt{\frac{\Sigma(X_1 - X)^2}{n-1}}$$

$$\text{S.D.} = \sqrt{\frac{2.9888}{n-1}} = \sqrt{\frac{2.9888}{11}} = \sqrt{0.2717} = 0.5214$$

TABLE II.—ASSAY OF MERBROMIN BY PRECIPITATION WITH THIOACETAMIDE

No.	Weight of Sample, Gm.	Weight of Precipitate, Gm.	% Weight of Mercury
1	0.2711	0.0807	25.66
2	0.2990	0.0899	25.92
3	0.3226	0.0970	25.92
4	0.3642	0.1086	25.72
5	0.2615	0.0775	25.56
6	0.2908	0.0861	25.53
7	0.3122	0.0957	26.42
8	0.3364	0.1010	25.89
9	0.2846	0.0852	25.22
10	0.2916	0.0876	25.90
11	0.2778	0.0834	25.88
12	0.3289	0.0984	25.79
			309.41
		Mean =	25.78

$$\text{S.D.} = \sqrt{\frac{\Sigma(X_1 - X)^2}{n-1}} = \sqrt{\frac{0.9277}{11}} = \sqrt{0.0833} = 0.2886$$

TABLE III.—ASSAY OF MERCURIC CHLORIDE BY PRECIPITATION WITH HYDROGEN SULFIDE GAS

No.	Weight of Sample, Gm.	Weight of Precipitate, Gm.	% Weight of Mercury
1	0.5122	0.4375	99.62
2	0.4968	0.4261	100.01
3	0.4664	0.3979	99.56
4	0.4912	0.4215	100.01
5	0.5244	0.4475	99.60
6	0.5218	0.4449	99.52
7	0.5190	0.4420	99.39
8	0.4758	0.4068	99.59
9	0.5324	0.4552	99.80
10	0.5010	0.4274	99.56
11	0.4883	0.4178	99.84
12	0.4439	0.3778	99.32
13	0.5103	0.4357	99.62
			1295.44
		Mean =	99.64

$$\text{S.D.} = \sqrt{\frac{\Sigma(X_1 - X)^2}{n-1}} = \sqrt{\frac{0.5353}{12}} = \sqrt{0.04460} = 0.2112$$

TABLE IV.—ASSAY OF MERCURIC CHLORIDE BY PRECIPITATION WITH THIOACETAMIDE

No.	Weight of Sample, Gm.	Weight of Precipitate, Gm.	% Weight of Mercuric Chloride
1	0.4822	0.4110	99.49
2	0.4618	0.3940	99.59
3	0.4929	0.4198	99.39
4	0.5446	0.4645	99.54
5	0.5003	0.4268	99.54
6	0.5118	0.4361	99.44
7	0.4432	0.3789	100.00
8	0.4681	0.3995	99.60
9	0.4924	0.4198	99.49
10	0.5100	0.4348	99.50
11	0.4223	0.3610	99.76
12	0.3886	0.3315	99.80
13	0.4216	0.3590	99.39
14	0.4108	0.3508	99.69
			1394.22
		Mean =	99.58

$$\text{S.D.} = \sqrt{\frac{\Sigma(X_1 - X)^2}{n-1}} = \sqrt{\frac{0.3868}{13}} = \sqrt{0.02975} = 0.1725$$

precipitation of metallic sulfides but should be as hot as possible without actually boiling.

EXPERIMENTAL

A comparison was made of the methods and results of the quantitative analysis of merbromin N.F. (9) and mercury bichloride U.S.P. (10) for mercury content, using hydrogen sulfide gas and thioacetamide solution.

The hydrogen sulfide gas was generated by the conventional method of adding dilute acid to iron sulfide and then charging the generators after each precipitation. Sufficient commercial, reagent grade thioacetamide was dissolved in distilled water to make a 1 *M* or 7.5% w/v solution, and it was stored in a dark amber-colored bottle. The solution was heated to approximately 80° before use to hasten hydrolysis of thioacetamide. Otherwise, the analysis of mercuric chloride was carried out in accordance with the United States Pharmacopeia (10); the analysis of merbromin was carried out in accordance with the National Formulary (9, 11).

Identical analyses were conducted for each of the two chemicals described above (see Tables I–IV), using thioacetamide wherever hydrogen sulfide was called for. The amount of thioacetamide required for the reaction was calculated on the basis of its hydrolysis product, hydrogen sulfide:

For mercuric chloride

$$HgCl_2 + H_2S \longrightarrow HgS + 2HCl$$
$$271.52 \qquad 34 \longrightarrow 232.68$$
$$CH_3CSNH_2 + H_2O \longrightarrow H_2S + CH_3CONH_2$$
$$75 \qquad 18 \longrightarrow 34$$

75 Gm. thioacetamide is needed to convert 271.52 Gm. of $HgCl_2$ to HgS, giving a gravimetric factor of 75/271.52 = 0.276.

COMPARISON BETWEEN TABLES I AND II[a]

	Table I	Table II
No. of analyses	12	12
Mean	25.73	25.78
S.D.	0.5214	0.2886

$$\text{S.D.D.} \sqrt{\frac{\sigma_1^2 + \sigma_2^2}{n_1 + n_2 - 2}} = \frac{0.5214 + 0.2886}{\sqrt{0.22}} = \frac{0.8100}{4.69} = 0.1727$$

$$\text{Computing } t \text{ from } \frac{\bar{X}_1 - \bar{X}_2}{\text{S.D.D.}} = \frac{0.05}{0.1727} = 0.2895$$

$t_{0.01}$ 22 *d.f.* = 2.819

[a] S.D. = standard deviation, S.D.D. = standard deviation of the difference, σ_1 and σ_2 = standard deviation of the arithmetic mean, $\bar{X}_1$ = mean of first group, $\bar{X}_2$ = mean of second group, and n = No. of analyses.

For merbromin

$$C_{20}H_8Br_2HgNa_2O_6 + H_2S \longrightarrow C_{20}H_8Br_2Na_2O_5 + HgS + H_2O$$
$$750.71 \qquad 34 \longrightarrow 232.68$$

75 Gm. thioacetamide is required for 750.71 Gm. of merbromin, giving a gravimetric factor of 75/750.71 = 0.0999.

A statistical evaluation (12) for the confidence limits for the two methods was made for both chemicals assayed. These tests indicate no significant difference of results between the use of hydrogen sulfide and thioacetamide in the analysis of mercuric chloride and merbromin.

COMPARISON BETWEEN TABLES III AND IV

	Table III	Table IV
No. of analyses	13	14
Mean	99.64	99.58
S.D.	0.2112	0.1725

$$\text{S.D.D.} \sqrt{\frac{\sigma_1^2 + \sigma_2^2}{n_1 + n_2 - 2}} = \frac{0.2112 + 0.1725}{\sqrt{25}} = \frac{0.3837}{5} = 0.0767$$

$$\text{Computing } t \text{ from } \frac{\bar{X}_1 - \bar{X}_2}{\text{S.D.D.}} = \frac{0.06}{0.0767} = 0.3395$$

$t_{0.01}$ 25 *d.f.* = 2.787

DISCUSSION

In the preceding analyses, it was found that thioacetamide could be used in place of hydrogen sulfide gas in the assay of merbromin and mercuric chloride. Results between the two methods agree fairly well. Thioacetamide solution is easier to use than hydrogen sulfide gas. Since, in the use of thioacetamide a moderate excess yielding, in turn, a moderate excess of hydrogen sulfide can be calculated quite accurately, there is very little danger of toxicity and hardly any unpleasant odor.

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Hydrolytic Stability of Calcium Acetylsalicylate Carbamide in the Presence of High Concentrations of Additives

By EUGENE L. PARROTT†

The hydrolysis of calcium acetylsalicylate carbamide in aqueous solution at 25, 40, and 50° in the pH range from 1 to 9 has been studied. The effect of high concentrations of several additives, commonly used in pharmaceutical formulation, on the rate of hydrolysis was determined.

NUMEROUS studies (1–5) of the stability of acetylsalicylic acid and its derivatives have been made over a wide range of pH and temperature; however, few reports have been quantitatively concerned with their hydrolytic stability in the presence of high concentrations of additives commonly used in pharmaceutical formulation.

In the development of dosage forms, not only the pH effect but other effects of the ingredients on the stability of a drug must be considered. The kinetics of degradation of many drugs have been studied as a function of pH or in the presence of a low concentration of additives. A limited number of studies have been conducted with additives utilized as stabilizers by virtue of complex formation; yet, even these studies have employed low concentrations of complexing agents (6–8). Practical formulations often contain large amounts of additives beyond the concentration range at which the laws of dilute solutions apply.

The purpose of this investigation was to study the kinetics of hydrolysis of calcium acetylsalicylate carbamide in the presence of a high concentration of several pharmaceutical additives.

Calcium acetylsalicylate carbamide,[1] a complex salt of urea, is an amorphous, white powder with a melting point of 243–245°, at which decomposition occurs. It has a molecular weight of 458. Its solubility made it particularly suitable for this work. At 37° it is soluble to the extent of 231 mg./ml. of solution, yielding a solution with a pH 4.8.

EXPERIMENTAL

Apparatus and Reagents.—Unless specified otherwise all chemicals were reagent grade. Aminoacetic acid N. F.; calcium acetylsalicylate carbamide (Calurin), Dorsey Laboratories; gelatin U. S. P.; glycerin, U. S. P.; polyethylene glycol (Carbowax 6000), Union Carbide and Carbon Co.; polyvinylpyrrolidone (Plasdone K 30), Antara Chemicals; sodium lauryl sulfate U. S. P.; sorbitol; 0.2 *M* ammonium chloride; 0.2 *M* ammonium hydroxide; 0.2 *M* boric acid; 0.1 *M* citric acid; 0.2 *M* disodium phosphate; 0.1 *N* hydrochloric acid; 0.5 *M* sodium borate; constant temperature bath; thermometer calibrated to 0.1°; Beckman pH meter; Beckman model DU spectrophotometer; silica cells.

Procedure.—Calcium acetylsalicylate carbamide, 0.250 Gm., was accurately weighed into a 250-ml. volumetric flask and dissolved in a sufficient amount of buffer to bring to a volume of 250 ml. of solution. The buffer solutions, ranging from pH 1 to 9, were preheated to the temperature at which the particular determinations were to be made. The flask was placed in the constant temperature bath and a 10-ml. sample, representing the zero-time sample, was withdrawn by pipet, transferred to a 100-ml. volumetric flask, and the volume was made to 100 ml. with 0.1 *N* hydrochloric acid. Absorbance of this solution was determined at 302 mμ, using a spectrophotometer with a blank of an identical system not containing calcium acetylsalicylate carbamide but diluted in the same manner. By means of a standard absorption curve, the amount of salicylic acid formed was measured and the concentration of calcium acetylsalicylate carbamide was calculated. Samples were removed at definite intervals of time and analyzed by this procedure. The method used is essentially that of Edwards (1).

McIlvaine's buffer was used from pH 3 to 6; Palitzsch's buffer was used from pH 7 to 9. At less than pH 3, Sorensen's citrate-hydrochloric acid buffer was employed. In the presence of polyhydric alcohols, an ammonium hydroxide-ammonium chloride buffer was used instead of Palitzsch's buffer to avoid the formation of a strongly ionized, complex acid (9). The additives were dissolved in the buffer and equilibrated to 40°. Any adjustments in pH were made using a Beckman pH meter model H. All concentrations are expressed in terms of per cent weight in volume.

RESULTS AND DISCUSSION

The rate of hydrolysis of calcium acetylsalicylate carbamide was followed over a pH range of 1 to 9 at 25, 40, and 50°. A plot of the logarithm of concentration against time produced a straight line

Received November 29, 1961, from the College of Pharmacy, University of Nebraska, Lincoln.
Accepted for publication January 5, 1962.
This project was supported in part by Dorsey Laboratories.
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[1] Marketed as Calurin by Dorsey Laboratories, a division of the Wander Co. and kindly supplied by them.

indicative of a pseudo first-order reaction with the net velocity being a function of several possible reactions (1, 3). Figure 1 is typical of the results obtained.

The observed velocity constants were determined and are plotted in Fig. 2. The velocity constants are expressed in reciprocal hours.

It was thought desirable to compare the stability of calcium acetylsalicylate carbamide under these conditions with the stability of acetylsalicylic acid.

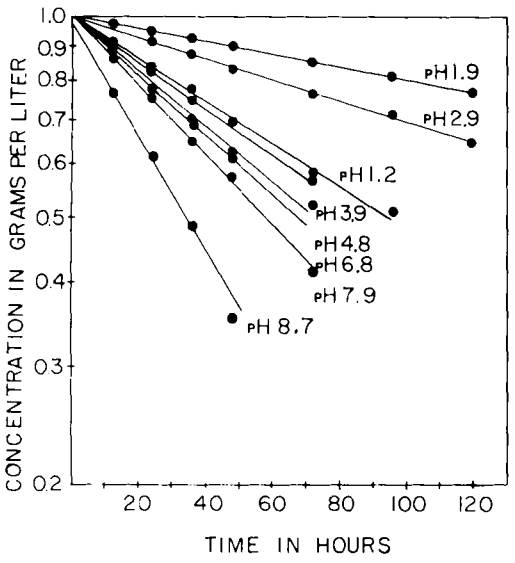


Fig. 1.—Pseudo first-order hydrolysis of calcium acetylsalicylate carbamide at 25°.

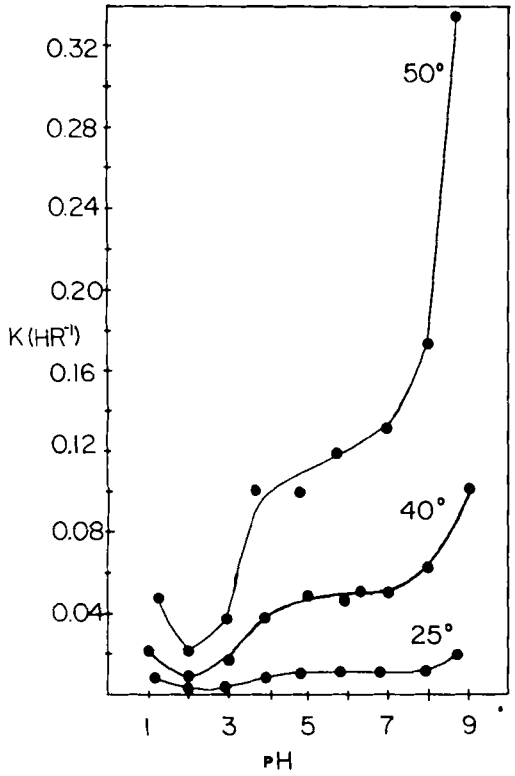


Fig. 2. -Observed velocity constants for calcium acetylsalicylate carbamide hydrolysis as a function of pH.

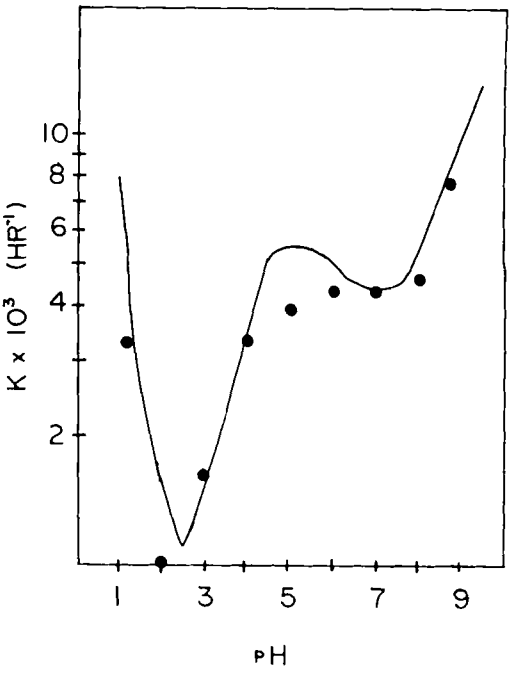


Fig. 3.—Observed velocity constants for aspirin and calcium acetylsalicylate carbamide hydrolysis at 17° as a function of pH. ——, drawn from Edwards, L., *Trans. Faraday Soc.*, **46**, 730(1950); ●, calculate K at 17° for calcium acetylsalicylate carbamide.

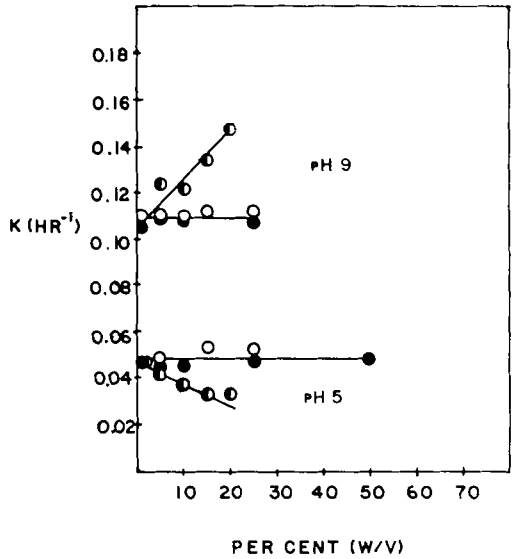


Fig. 4.– Observed velocity constants for calcium acetylsalicylate carbamide hydrolysis at 40° as a function of concentration of polyethylene glycol 6000, polyvinylpyrrolidone, and sodium lauryl sulfate. ●, Polyethylene glycol 6000; ○, polyvinylpyrrolidone; ◐, sodium lauryl sulfate.

By the use of the Arrhenius equation, the energy of activation was determined and the velocity constants for calcium acetylsalicylate carbamide at 17° were calculated for comparison with the work of Edwards. An examination of Fig. 3 shows that within experimental error, the hydrolytic stability of acetylsalicylic acid and calcium acetylsalicylate carbamide are identical. At 25° the velocity constant for the hydrolysis of calcium acetylsalicylate carbamide was the same as that of acetylsalicylic acid as determined by Garrett (3).

Bolton (4) has shown that although the pH of an acetylsalicylic acid solution is the major consideration in determining the rate of hydrolysis, certain additives, such as a solution of sodium citrate and phosphate, increase degradation. A similar effect was found at a pH 9 with a change of buffer in a solution of calcium acetylsalicylate carbamide. As shown in Figs. 4 and 6, at pH 9 the velocity constant is 0.11 hr.$^{-1}$ and 1.44 hr.$^{-1}$ in Palitzsch's buffer and ammonium hydroxide-ammonium chloride buffer, respectively. This variation presents another example of the necessity of considering the additives, *per se*, as well as the pH the additives maintain.

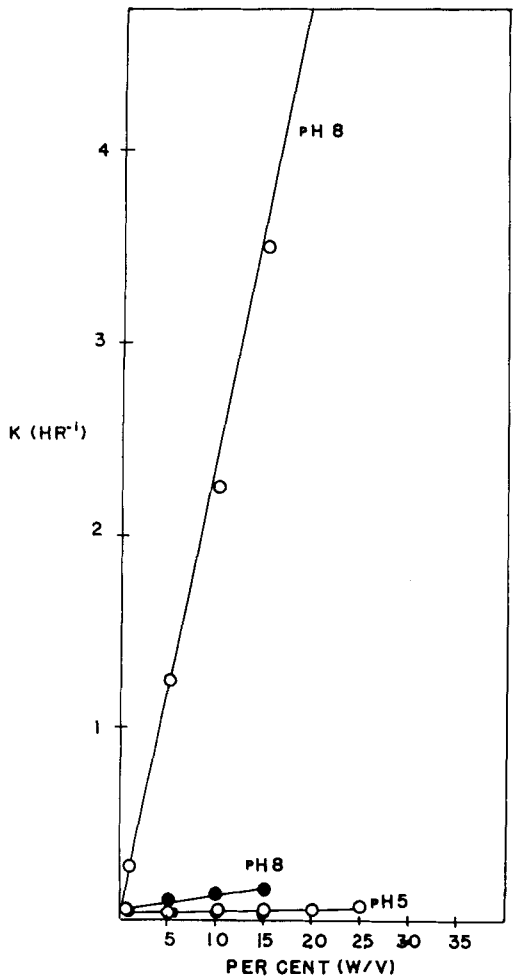


Fig. 5.—Observed velocity constants for calcium acetylsalicylate carbamide hydrolysis at 40° as a function of concentration of glycine and gelatin. O, Aminoacetic acid; ●, gelatin.

Also, it is shown in Fig. 5 that aminoacetic acid increased the rate of hydrolysis of calcium acetylsalicylate carbamide. Thus, if one attempted to buffer a calcium acetylsalicylate carbamide solution at an acidic pH using Sorensen's glycocoll-hydrochloric acid buffer, the resulting formulation would be less stable than a similar product buffered to the same pH, using McIlvaine's buffer.

It is the purpose of this work to observe, at an acidic and an alkaline pH, the rate of hydrolysis as a function of the concentration of common pharmaceutical ingredients; therefore, no attempt has been made to maintain a given ionic strength but the additives were added as they might be in actual developmental procedures.

Nonionic Polymers.—Polyethylene glycol 6000 and polyvinylpyrrolidone were chosen as examples of polymers frequently used in compounding pharmaceuticals. From 0.1 to 50% polyethylene glycol 6000 was added to calcium acetylsalicylate carbamide solutions buffered at pH 5 and 9 at 40°. The observed velocity constants were determined at each concentration and plotted in Fig. 4. Polyethylene glycol 6000 in concentrations up to 50% had no effect on the hydrolytic rate of calcium acetylsalicylate carbamide at pH 5 and 9 at 40°.

Similar experimentation with polyvinylpyrrolidone in concentrations from 1 to 25% showed no effect on the rate of hydrolysis of calcium acetylsalicylate carbamide.

It has been reported that polyethylene glycols retard the hydrolysis of acetylsalicylic acid solutions (10). Although the concentrations of these polymers were high, the mole fractions of the polymers were small. It may be for this reason that no stabilization was observed in this work and it would appear that to have any value as a stabilizer of solutions of acetylsalicylic acid or its derivatives, the polymers should completely replace water in a liquid preparation.

Polyols.—The use of sugar and glycerin has been suggested as a means of retarding the hydrolysis of solutions of aspirin (11, 12). The wide-spread use

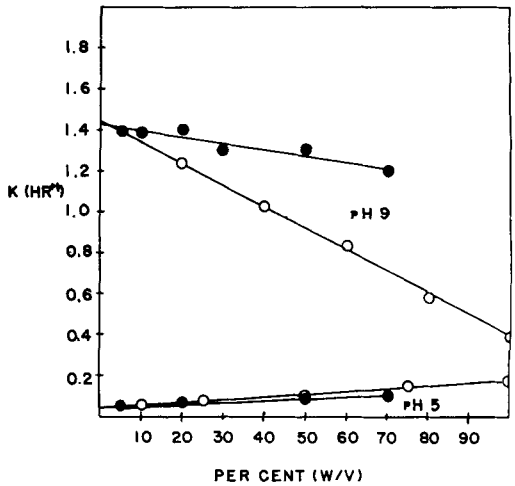


Fig. 6.—Observed velocity constant for calcium acetylsalicylate carbamide hydrolysis at 40° as a function of concentration of polyols. ●, Glycerin; O, sorbitol.

of sorbitol in liquid pharmaceuticals suggested that sorbitol be investigated in regard to its effect on the hydrolysis of calcium acetylsalicylate carbamide. As shown in Fig. 6, at pH 9 an increase in the concentration of glycerin or sorbitol decreased the rate of hydrolysis. With 70% sorbitol, the half-life was increased approximately 15%; with 100% glycerin, the half-life was increased almost threefold. This is in agreement with other workers who suggested that sorbitol and glycerin may bind water tenaciously so the water is less available for chemical interaction, thus enhancing the stability of the preparation (13).

At pH 5 the rate of hydrolysis increased as the concentration of glycerin or sorbitol increased. At pH 5, the half-life of calcium acetylsalicylate carbamide in 70% sorbitol decreased approximately one-half; in 100% glycerin, the half-life decreased to roughly one-third. It is difficult to rationalize this behavior in view of the above statement. According to the available water theory, a given concentration of sorbitol or glycerin should bind the same amount of water in both alkaline and acidic medium. The fact that this is not actually the case suggests that some other mechanism is operative.

Amphoteric Amines.—Aminoacetic acid and gelatin were chosen as typical amphoteric substances that might be used in pharmaceutical formulation. As shown in Fig. 5, an increase in aminoacetic acid concentration in both alkaline and acidic medium increased the rate of hydrolysis. At pH 5, the addition of 25% aminoacetic acid decreased the half-life of calcium acetylsalicylate carbamide approximately 40%; at pH 8, the addition of 25% aminoacetic acid decreased the half-life almost tenfold.

At pH 5, there was little significant change in the stability of calcium acetylsalicylate carbamide upon the addition of up to 15% gelatin. At a pH 8, the increase in gelatin concentration caused an increase in the rate of hydrolysis; 15% gelatin produced approximately a 60% decrease in the half-life.

It appears that protein-type additives decrease the hydrolytic stability of calcium acetylsalicylate carbamide in both alkaline and acidic solutions.

Surfactant.—Anionic surface-active agents are occasionally used as aids to facilitate tablet disintegration, as wetting agents, and as dispersing agents. Although the amounts employed are small, it seemed advisable to determine the effect of a typical anionic surfactant, such as sodium lauryl sulfate, on the hydrolysis of calcium acetylsalicylate carbamide. At pH 5, it was found that an increase in the concentration of sodium lauryl sulfate retarded hydrolysis; 20% sodium lauryl sulfate increased the half-life approximately one-third. At pH 9, 20% sodium lauryl sulfate produced approximately 30% *reduction* in half-life.

One might postulate that in acidic medium the surfactant is in the highly polar form of a sulfuric acid derivative. This strong dipole may decrease the activity of the water, thus slowing the rate of hydrolysis. In alkaline medium, the salt form of the surfactant may have less affinity for water, resulting in a greater activity for water and a more rapid hydrolysis in alkaline solution.

SUMMARY

1. The hydrolytic kinetics of calcium acetylsalicylate carbamide were studied over a pH range from 1 to 9 at 25, 40, and 50°.

2. In evaluating the stability of a solution, the effect of the constituents *per se* should be evaluated as well as the pH effect.

3. In acidic solution, aminoacetic acid, glycerin, and sorbitol increased the rate of hydrolysis of calcium acetylsalicylate carbamide solutions.

4. In alkaline solution, aminoacetic acid, gelatin, and sodium lauryl sulfate increased the rate of hydrolysis of calcium acetylsalicylate carbamide solutions.

5. In alkaline solution, glycerin and sorbitol decreased the rate of hydrolysis of calcium acetylsalicylate carbamide solutions.

6. In acidic solutions, sodium lauryl sulfate decreased the rate of hydrolysis of calcium acetylsalicylate carbamide solutions.

7. In both acidic and alkaline solution, polyethylene glycol and polyvinylpyrrolidone had no effect on the rate of hydrolysis of calcium acetylsalicylate carbamide solutions.

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Anticancer Evaluation of *Amphipterygium adstringens*

By EDWARD E. GONZALEZ†, GEORGE F. McKENNA‡, and JAIME N. DELGADO

The antineoplastic activity of numerous *Amphipterygium adstringens* fractions has been studied on dba mice bearing RC mammary adenocarcinoma. Methods of anticancer evaluation are fully described. Several tumor inhibitors, as well as activators, were found in the various drug fractions. The methanol extract of the whole drug exhibited the most pronounced inhibitory activity. The tumor-damaging capacity of the drug has been shown to be mainly associated with the saponin-containing fractions.

THE POSSIBILITIES of discovering natural products with anticancer activity have been the motivation for establishing and maintaining many screening programs in cancer laboratories throughout the country. Although research in this area is mainly concerned with negative data, it is hoped that reports of this nature will spare others the time and effort of investigating sources which have proved to be of no value, and secondly, will focus attention on substances that are worthy of further exploration. Little progress has been reported in the field of cancer chemotherapy and promising leads are still as scarce today as they were several years ago (1, 2).

Several laboratories with cancer screening programs have reported the testing of plant extracts for possible adverse tumor effects. Belkin and associates have published a series of papers on the tumor-damaging action of numerous plant preparations (3–5). Faust and coworkers have reported in some detail the antineoplastic activity of *Citrullus colocynthis* on Sarcoma-37 (6). More recently, McKenna, *et al.* (7), examined numerous essential oils as potential antitumor agents. Testing and screening materials for anticarcinogenic activity have been routine in the Clayton Foundation Biochemical Institute cancer research laboratories for a number of years (8–12).

This study was initiated after preliminary screening tests showed that *Amphipterygium adstringens* Schlecht, fam. *Julianiaceae* possessed a certain degree of anticancer activity in both mice and embryonated eggs (9). Since *Amphipterygium adstringens* was readily available in ufficient quantity, a simultaneous chemical investigation was conducted on the drug in an effort to ascertain the nature of the component responsible for the antineoplastic activity (13).

Received December 6, 1961, from the College of Pharmacy, Division of Pharmaceutical Chemistry, University of Texas, Austin 12.

Accepted for publication January 5, 1962.

This paper is abstracted in part from a thesis submitted to the Graduate School of The University of Texas by Edward E. Gonzalez in partial fulfillment of the requirements for the degree of Master of Science.

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Presented to the Scientific section, A.PH.A., Las Vegas, meeting, April 1962.

EXPERIMENTAL

Fractionation of Drug.—The drug fractions used in this study were essentially those isolated during the course of the phytochemical investigation (13). The various drug fractions that were isolated and prepared for this study are schematically outlined in Fig. 1.

Preparation of Drug Fractions.—The drug fractions were stored in the refrigerator and processed for testing as soon as possible to avoid possible loss of biological activity. Before the drug fractions were tested biologically, they were sterilized by one of two methods. Water-soluble extracts were sterilized by filtering through a Selas No. 2 porcelain filter. Other extracts were sterilized by placing the material in a sterile Erlenmeyer flask containing a small quantity of 70% alcohol. The flask was placed in a desiccator under aseptic conditions and the alcohol evaporated by use of a vacuum pump. Acetone-soluble extracts were tested by the latter method with the exception that acetone was used in place of 70% alcohol. The extracts were then checked for sterility according to procedures described in previous reports (9). Sterile extracts were tested for toxicity by trials in mice. Subdermal injections of various concentrations were made and the effect produced on weight and visceral organs was noted. It was important to select a dosage range that would give distinguishable results between possible toxic reactions and tumor damage.

Type of Neoplasms.—The fractions were tested for antineoplastic activity against RC mammary adenocarcinoma in dba mice. This mammary tumor has been employed for screening purposes in other cancer research laboratories (14). Transplants of RC mammary adenocarcinoma in the dba strain have been maintained in the Clayton Foundation Biochemical Institute cancer laboratories since the time of their spontaneous development in 1941. The preliminary screening tests were conducted on C_3H mouse mammary carcinoma cultivated in eggs (15), since the time and effort involved is much less than that required by the animal transplant method.

Methods of Anticancer Evaluation.—The method described for the anticancer evaluation studies is somewhat similar to that reported earlier by

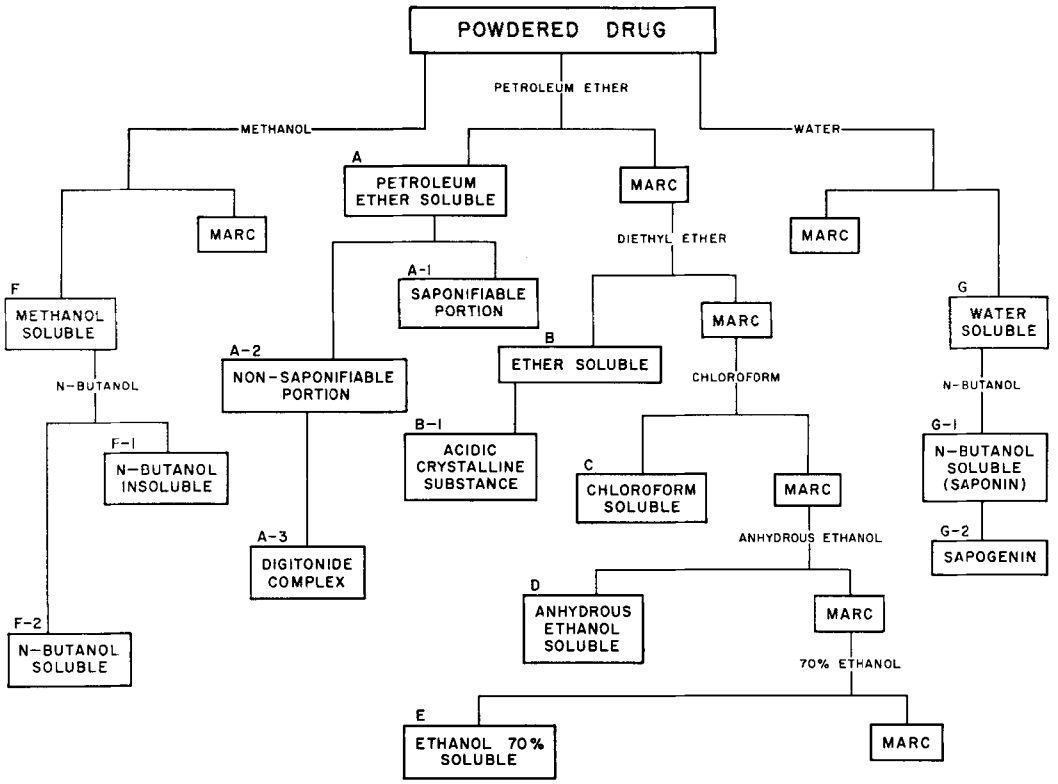


Fig. 1.—Fractionation of *Amphipterygium adstringens*.

McKenna and co-workers (9, 11). The dba mice used in this study were kept in stock at least 10 days before use and were maintained on regular laboratory chow and water, *ad libitum*. Transplants of the RC mammary adenocarcinoma were made by subdermal injections of sterile tumor suspensions. Fresh homogenized tumor tissue (0.02 ml.) dispersed in 0.2 ml. of 0.85% saline solution was injected in the right inguinal area. The mice were then divided randomly into equal groups. One group was kept as control, and received in the left inguinal area an injection of 0.2 ml. of sterile water for injection. The remaining mice, designated as the experimental group, received daily injections of the test fraction for 7 days. The test fractions were likewise administered in the left inguinal region, in the form of either aqueous solutions or suspensions, 72 hours after tumor implantation. All of the test fractions were used in concentrations of 750 mcg./ml. Only during the preliminary experiments were saturated solutions near maximum tolerated doses employed. All the animals were sacrificed on

Table I.—Antitumor Activity of Some *Amphipterygium adstringens* Extracts in dba Mice Bearing RC Mammary Adenocarcinoma

Type of Extract	Dosage Form	No. of Mice Used	Av. Body Weight at Autopsy, Gm.	Av. Tumor Weight at Autopsy Gm.	Percentage Body Growth at Autopsy[a]	Percentage Tumor Growh at Autopsy[a]
A	Suspension	12	22.80	0.47	102	157
A-1	Suspension	10	25.10	0.77	97	70
A-2	Suspension	12	27.10	0.95	105	86
A-3	Solution	8	23.10	0.51	104	68
B	Suspension	12	26.50	0.78	103	70
B-1	Solution	12	19.80	0.38	89	127
C	Suspension	12	22.80	0.64	102	213
D	Solution	12	24.10	0.40	109	53
E	Solution	12	22.30	0.30	97	60
F	Solution	16	25.70	0.51	100	46
F-1	Solution	12	20.30	0.40	91	133
F-2	Solution	8	23.60	0.47	102	50
G	Solution	10	24.40	0.42	95	52
G-1	Solution	10	22.30	0.38	92	51
G-2	Solution	10	26.00	1.10	101	100

[a] Control equals 100%.

the eleventh day of tumor growth and the weights of the animals and tumors recorded. The effectiveness of the drug fractions was evaluated on the basis of the difference in the average weight of the tumor in the experimental group as compared with the tumor growth in the control animals.

RESULTS AND DISCUSSION

The experimental results are given in Table I. It will be noted from the data shown that the methanol extract (F) of the whole drug exhibited the greatest amount of inhibition. This particular extract (F) induced a 54% decrease in tumor growth as compared with controls. Comparable inhibitory activity was exhibited by the *n*-butanol fraction (F-2) of the methanol extract (F). The F-2 fraction evinced a 50% decrease in tumor growth as compared with controls. In general, the most significant inhibitory activities were demonstrated by fractions D, E, F, F-2, G, and G-1. In addition to these inhibitory fractions, growth activators were found among the various drug fractions. Tumor growth was stimulated primarily by the petroleum ether (A) and chloroform (C) extracts of the drug. The petroleum ether extract (A) increased tumor growth by 57% as compared with controls. The chloroform extract (C) accelerated and increased tumor growth by as much as 113%. Body weight was not affected by the various fractions, with the possible exception of the acidic crystalline substance (B-1) isolated from the ether extract (13). The B-1 treated animals experienced an average body weight loss of 4.21 Gm. as compared with the average body weight of all other treated animals.

None of the experimental animals showed any toxic drug symptoms. However, upon autopsy, it was noted that some of the experimental animals did have irritation at the site of drug release and that, in a few cases, the drug was not thoroughly dispersed and absorbed. These manifestations were mainly associated with those fractions (A, A-1, A-2, B) which were administered in the form of suspensions.

Since the inhibitory activities of the saponin-containing fractions (D, E, F, F-2, G) were comparable to that of the isolated saponin (G-1), it is suggested that the anticarcinogenic action of *Amphipterygium adstringens* is primarily attributed to the presence of the steroidal saponin. Although this generalization is well supported by the fact that the saponin-exhausted fraction (F-1) exhibited a loss of inhibitory activity, it is rather difficult to justify completely the inhibitory activity on this observation since saponins are well known for their characteristic hemolytic activity, and yet no deleterious effects were observed. It should be noted also that the aglycon fraction (G-2) possessed no biological activity. Compared with the saponin (G-1), the aglycon fraction (G-2) lost inhibitory activity by 49%. These data indicate that the carbohydrate residues may play an important role in the biological action of the steroidal saponin. It may well be that the carbohydrate residues significantly affect absorption and distribution of the agent, as in the case of the cardioactive glycosides.

SUMMARY

Over 150 dba mice bearing RC mammary adenocarcinoma were used to study the antineoplastic effect of *Amphipterygium adstringens*, Schlecht. Numerous drug fractions were prepared and evaluated for anticancer activity. The results of this investigation are summarized.

1. The methanol extract (F) of the whole drug exhibited the most pronounced inhibitory activity. A 54% decrease in tumor growth was induced by the crude extract as compared with controls

2. Tumor growth was stimulated by the petroleum ether (A) and chloroform (C) extracts of the drug.

3. The saponin-containing fractions (D, E, F, F-2, G) exhibited inhibitory activity comparable to the isolated saponin (G-1).

4. The saponin-exhausted fraction (F-1) showed complete loss of inhibitory activity.

5. In contrast to the saponin (G-1), the aglycon (G-2) evinced no biological activity. Compared with the saponin (G-1), the aglycon (G-2) lost inhibitory activity by 49%.

6. Body weight was affected only by the acidic crystalline substance (B-1) isolated from the ether extract. An average body weight loss of 4.21 Gm., as compared with other treated animals, was noted.

7. The experimental results indicate that the anticarcinogenic activity of *Amphipterygium adstringens* may be due to the presence of the steroidal saponin (G-1).

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Isotonic Solutions XII

Permeability of Red Corpuscles to Various Water-Soluble Organic Iodine Compounds

By LORNE A. SCHNELL† and WILLIAM J. HUSA

In the present investigation, inquiry was made into the penetration effects of certain water-soluble organic iodine compounds with respect to the human erythrocyte membrane. Employing the hemolytic method, van't Hoff factors were calculated for each compound. The results indicated that all compounds studied yielded hemolytic *i* values which were higher than *i* values obtained by the freezing point method. Experiments were also conducted in the presence of 0.2% sodium chloride; in some cases this had the effect of lowering the hemolytic *i* values. Some tests were made in which the defibrinated blood used in determination of hemolytic *i* values was replaced by whole blood containing heparin sodium. The *i* values obtained with heparinized blood were in agreement with the *i* values resulting from the use of defibrinated blood. None of the compounds studied appeared to cause hemolysis of erythrocytes by penetration of the cell; hemolysis was noted only in hypotonic solutions of these compounds.

PREVIOUS investigations (1–10) have dealt with the permeability of erythrocytes to a variety of chemical compounds. The purpose of the present study was to determine the permeability of erythrocytes to various water-soluble organic iodine compounds used as contrast media in roentgenographic diagnostic procedures. This class of compounds is administered parenterally in relatively high concentrations. No references were found in the literature on the permeability of erythrocytes to any of the compounds used in the present study. Hence, experiments were conducted to determine the hemolytic *i* values of some of the water-soluble organic iodine compounds; *i* values were also determined by the freezing point method.

EXPERIMENTAL

Collection of Blood.—In experiments utilizing defibrinated blood, samples were obtained and treated in precisely the same manner as described by Husa and co-workers (1–10). In experiments requiring heparinized blood, the sample was obtained in an identical fashion and immediately added to a 10-ml. graduated centrifuge tube which contained 0.05 ml. of heparin sodium solution, 10,000 units per ml., such that the 10-ml. heparinized blood sample contained 50 units of heparin sodium per ml. The centrifuge tube was inverted several times to insure thorough mixing, and the heparinized sample was then transferred to a 50-ml. Erlenmeyer flask and treated in the same manner as defibrinated blood.

Samples were obtained principally from the forearm veins of a 27-year old white male. On several occasions samples were generously donated by volunteers.

Preparation of Solutions.—Solutions of compounds which were available as water-soluble salts were prepared in essentially the same manner as described by other workers (1–10). Iodipamide sodium, iodipamide methylglucamine, and diatrizoate methylglucamine solutions were prepared by dissolving a quantity of the free acid in a solution containing an equimolar quantity of the proper base.

Calculation of Hemolytic *i* Values.—Quantitative determinations of per cent hemolysis and calculations of hemolytic *i* values were carried out in the manner described by Grosicki and Husa (2).

Chemicals.—All organic iodine compounds used in the investigation were generously contributed by their manufacturers: sodium diprotrizoate, sodium acetrizoate, and sodium iodohippurate by Mallinckrodt Chemical Works; sodium methiodal, iodopyracet, and sodium diatrizoate by Sterling-Winthrop Research Institute; iodipamide, diatrizoic acid, and methylglucamine by Squibb Institute for Chemical Research.

Freezing Point Determinations.—The apparatus used to determine the freezing points was that of Bartley (11), as modified by Husa and Adams (1). van't Hoff *i* factors were calculated according to the formula

$$\Delta T_f = K_f i m$$

where ΔT_f is the change in the freezing point of water after the addition of solute, K_f is the molal freezing point constant for water (1.86), i is the van't Hoff factor, and m is the molality of the solution.

Results with Heparinized Blood.—Some investigators (12) have used heparinized blood in experiments on isotonicity of solutions. Hence, the question arose as to whether results obtained with heparinized blood would be comparable with those using defibrinated blood. To throw light on this question, hemolytic *i* values were determined for the 10 compounds listed in Table I using the same procedure as before except that defibrinated blood was replaced by whole blood containing 50 units/ml. of heparin sodium. The *i* values obtained with

Received May 26, 1961, from the College of Pharmacy, University of Florida, Gainesville.
Accepted for publication December 11, 1961.
This paper is based in part upon a dissertation presented by Lorne A. Schnell to the Graduate Council of the University of Florida in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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TABLE I.—VALUES OF *i* FOR VARIOUS ORGANIC IODINE COMPOUNDS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75 PER CENT HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

Name of Compound	Hemolysis, % 25	50	75	Averages
Sodium diprotrizoate	2.34	2.40	2.54	2.43
Sodium diprotrizoate[b]	2.42	2.51	2.62	2.52
Sodium methiodal	1.93	1.94	1.98	1.95
Sodium methiodal[b]	1.99	2.03	2.07	2.03
Sodium acetrizoate	2.25	2.34	2.50	2.36
Sodium acetrizoate[b]	2.19	2.22	2.32	2.24
Sodium iodohippurate	2.15	2.19	2.27	2.20
Sodium iodohippurate[b]	2.15	2.17	2.23	2.18
Sodium iodomethamate	3.62	3.72	3.97	3.77
Sodium iodomethamate[b]	3.45	3.49	3.58	3.51
Iodipamide sodium	4.06	4.35	4.70	4.37
Iodipamide sodium[b]	3.73	3.84	3.94	3.84
Iodipamide methylglucamine	3.78	4.46	4.84	4.36
Iodipamide methylglucamine[b]	2.96	3.03	3.20	3.06
Iodopyracet	2.01	2.12	2.41	2.18
Iodopyracet[b]	1.61	1.61	1.64	1.63
Sodium diatrizoate	2.25	2.32	2.51	2.36
Sodium diatrizoate[b]	2.09	2.13	2.16	2.13
Diatrizoate methylglucamine	2.37	2.49	2.73	2.53
Diatrizoate methylglucamine[b]	2.48	2.54	2.75	2.59

[a] All *i* values represent an average of two blood samples.
[b] Values determined in the presence of 0.2% sodium chloride.

TABLE II.—VALUES OF *i* OF VARIOUS ORGANIC IODINE COMPOUNDS CALCULATED FROM FREEZING POINT DATA OBTAINED WITH A MODIFIED BARTLEY APPARATUS

Compound	Molality of Solution	Average Freezing Point Depression	*i* Value
Sodium diprotrizoate	0.025	0.087	1.87
Sodium methiodal	0.025	0.085	1.83
Sodium acetrizoate	0.025	0.089	1.91
Sodium iodohippurate	0.025	0.091	1.95
Sodium iodomethamate	0.025	0.138	2.97
Iodipamide sodium	0.025	0.138	2.97
Iodipamide methylglucamine	0.025	0.137	2.94
Iodopyracet	0.025	0.077	1.66
Sodium diatrizoate	0.025	0.091	1.95
Diatrizoate methylglucamine	0.025	0.093	2.00

heparinized blood agreed closely with the *i* values which resulted from the use of defibrinated blood, the differences being within experimental error.

DISCUSSION

The values of *i* obtained by the hemolytic method for the organic iodine compounds were consistently higher than the corresponding *i* values obtained by the freezing point method (see Table II). In solutions of some substances, erythrocytes lose electrolytes from within the cell, thus causing a decrease in internal osmotic pressure and a corresponding ncrease in resistance to hemolysis (2). Possibly such exosmosis from erythrocytes may occur in solutions of the organic iodine compounds, causing the higher *i* values obtained experimentally.

The difference between the hemolytic *i* value and the *i* value obtained by the freezing point method was smallest with sodium methiodal; in this case the hemolytic *i* value was 1.95 and the *i* value by the freezing point method was 1.83. In this connection it is of interest to note that sodium methiodal was the only nonaromatic compound used in this investigation.

The exosmosis from erythrocytes in solutions of some substances can be corrected in some cases by addition of a small proportion of sodium chloride (2). The presence of 0.2% sodium chloride reduced the hemolytic *i* values of some of the compounds tested, *e.g.*, sodium iodomethamate, iodipamide sodium, and iodipamide methylglucamine. In case of iodopyracet the hemolytic *i* value was reduced from 2.18 to 1.63 by the presence of 0.2% sodium chloride; the latter value agreed closely with the *i* value of 1.66 obtained by the freezing point method. For some of the other compounds, the abnormally high values of *i* by the hemolytic method were not reduced by the presence of 0.2% sodium chloride.

SUMMARY

1. Hemolytic *i* values obtained for various organic iodine compounds were consistently higher than the corresponding *i* values obtained by the freezing point method. In some, but not all, cases the hemolytic *i* values were lowered by the presence of 0.2% sodium chloride.

2. The organic iodine compounds tested apparently do not penetrate the human erythrocyte.

3. Hemolytic *i* values obtained for the organic iodine compounds using defibrinated blood were in agreement with similar values obtained with heparinized blood.

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Ergotamine Tartrate U.S.P.

By G. L. SZENDEY

On the basis of melting point and solubility, ergotamine tartrate U.S.P. is a mixture of ergotamine tartrate and ergotamine bitartrate. Several samples with different properties were studied by means of titrimetric methods in nonaqueous media using 0.05 *N* perchloric acid and 0.1 *N* potassium methoxide. The melting point well indicates the approximate ratio of ergotamine tartrate-bitartrate mixture. Ergotamine tartrate with a melting point of about 180° (decompn.) contains about 83% ergotamine base, equivalent to 94% ergotamine tartrate.

SEVERAL authors reported (1–4) a discrepancy concerning ergotamine tartrate U.S.P. It was stated that requirements for solubility and melting point represent, contrary to the British Pharmacopoeia, a mixture of ergotamine tartrate and ergotamine hydrogen tartrate (ergotamine bitartrate).

Table I lists the differing requirements of the British Pharmacopoeia and the United States Pharmacopeia of ergotamine tartrate.

The present paper reports on quantitative studies of ergotamine tartrate samples with different melting points and discusses questions deriving from the above-mentioned problem.

METHOD

Apparatus.—Microburets, 3 or 5 ml. Melting range apparatus with light paraffin bath and calibrated thermometer (type II, U.S.P. XVI).

Assay of Ergotamine Base in Ergotamine Tartrate

Reagents.—All reagents are analytical grade. *Glacial acetic acid*, Merck. *Anhydrous acetic acid* (1000 ml. of glacial acetic acid mixed with 60 ml. of acetic anhydride). *Crystal violet indicator*, 0.25% in anhydrous acetic acid. *Perchloric acid*, 0.05 *N*, prepared by mixing 5.5 ml. (8.4 Gm.) of perchloric acid (60%, d. 1.54) with 200 ml. of anhydrous acetic acid and 20 ml. of acetic anhydride; cooling the solution, then adding anhydrous acetic acid to make 1000 ml. The normality factor was obtained by titration against sym.-diphenylguanidine (mol. wt. 211.26, m.p. 147–149): approximately 20 mg., accurately weighed, was dissolved in 15 ml. of anhydrous acetic acid, 1–2 drops of crystal violet indicator was added and titrated with the perchloric acid solution until the violet color changed to blue-bluish green. Each 10.56 mg. of sym.-diphenylguanidine is equivalent to 1 ml. of 0.05 *N* perchloric acid.

Procedure.—About 70 mg. of ergotamine tartrate, accurately weighed, was dissolved in 15 ml. of anhydrous acetic acid, 1–2 drops of crystal violet indicator was added and titrated with 0.05 *N* perchloric acid until the violet color changed to blue-bluish green. Each 32.84 mg. of ergotamine tartrate [mol. wt. 1313.46, $(C_{33}H_{35}N_5O_5)_2.C_4H_6O_6$] or each 29.08 mg. of ergotamine base (mol. wt. 581.65, $C_{33}H_{35}N_5O_5$) is equivalent to 1 ml. of 0.05 *N* perchloric acid. Accuracy, ±0.5%.

Assay of Tartaric Acid in Ergotamine Tartrate

Reagents.—All reagents are analytical grade. *Anhydrous methanol. Potassium metal. Benzene*, dried over potassium metal. *Pyridine. Naphtholbenzein* (Merck) indicator, 1% in pyridine. *Potassium methoxide*, 0.1 *N*, prepared as follows: about 4.3 Gm. of freshly cut potassium metal was added, in small portions, to 150 ml. of ice-cooled anhydrous methanol contained in a 1000-ml. volumetric flask. When dissolved, sufficient benzene was added to make 1000 ml. The solution was preserved in a container, well protected from light and moisture. Standardization was made by titration against benzoic acid, Merck (mol. wt. 122.12): approximately 25 mg., accurately weighed, was dissolved in 15 ml. of neutralized pyridine, 1 drop of naphtholbenzein indicator was added and titrated with the potassium methoxide solution until the yellow color changed to green. Each 12.21 mg. of benzoic acid is equivalent to 1 ml. of 0.1 *N* potassium methoxide.

Procedure.—Ergotamine tartrate, 100–150 mg., accurately weighed, was dissolved in 15 ml. of neutralized pyridine, 3 drops of naphtholbenzein indicator was added and titrated with 0.1 *N* potassium methoxide until the yellow color changed to green. Protect the solution from the carbon dioxide of the atmosphere. Each 7.504 mg. of tartaric acid is equivalent to 1 ml. of 0.1 *N* potassium methoxide. Accuracy of the method, ±1.0%.

RESULTS

Analytical data of assays are reported in Table II. Estimation of ergotamine base was carried out in anhydrous acetic acid by using 0.05 *N* perchloric acid. Each value presented is the average of four parallel analyses. Evidently there is a large scale of possibilities between ergotamine tartrate and bitartrate to produce mixtures of both salts, where melting point indicates the approximate ratio.

Received January 27, 1962, from Research Laboratories, Sanol-Arzneimittel Dr. Schwarz GmbH., Monheim bei Düsseldorf, Bundesrepublik Deutschland.
Accepted for publication February 26, 1962.

TABLE I.—COMPARISON OF REQUIREMENTS, B.P. AND U.S.P.

Properties	B.P. 1953	B.P. 1958	U.S.P. XIV	U.S.P. XV	U.S.P.XVI
Solubility	Soluble in water, forming a solution which is liable to become turbid; the addition of tartaric acid is necessary to maintain a clear solution. Soluble in 500 parts of alcohol (90%).		1 Gm. of ergotamine tartrate dissolves in about 500 ml. of water and in about 500 ml. of alcohol.		
Melting point	Softens at about 187° and decomposes at about 192° without melting.		Melts between 177 and 184° (decompn.)		Melts at about 180° (decompn.)

TABLE II.—PROPERTIES OF ERGOTAMINE TARTRATE SAMPLES

Substance	Solubility	M.P., °C.	Contents of Alkaloid Calcd.,[a] % Ergotamine Base Found	Ergotamine Base Theory	Ergotamine Tartrate Found	Ergotamine Tartrate Theory
Ergotamine tartrate B.P.	As B.P. standard	190–193 (decompn.)	88.08	88.57	99.44	100.00
Ergotamine tartrate U.S.P.	As U.S.P. standard	179–180 (decompn.)	82.02	88.57	93.75	100.00
Ergotamine bitartrate-ergotamine tartrate B.P. (6:4)	As U.S.P. standard, ergotamine tartrate	177–180 (decompn.)	82.68	83.12[b]	93.35	93.85[b]
Ergotamine bitartrate	Soluble in about 500 ml. of water and in about 500 ml. of alcohol (90%)	173–175 (decompn.)	78.38	79.49	88.50[c] 98.60[d]	89.75[c] 100.00[d]

[a] All values are calculated on dried substance (100° *in vacuo*). [b] Based on a mixture of ergotamine bitartrate-ergotamine tartrate (6:4). [c] Calculated on ergotamine tartrate (equivalent wt. = mol. wt./2) = 656.73). [d] Calculated on ergotamine bitartrate (equivalent wt. = mol. wt. = 731.74). 10.25% is the difference between the two equivalent weights. Ergotamine bitartrate was prepared from ergotamine base by precipitation in methanol using a surplus of tartaric acid (about 30% of the base quantity). This salt is official in the "Pharmacopoea Bohemoslovenica," 2nd ed., 1954.

Further data have been obtained by analyzing, on one hand, the contents of ergotamine base, on the other, the contents of tartaric acid of samples with different melting points (Table III). The

TABLE III.—ASSAY OF ERGOTAMINE TARTRATE SAMPLES[a]

M.P., °C.	Ergotamine Base, % Found	Ergotamine Base, % Theory	Tartaric Acid, % Found	Tartaric Acid, % Theory	Total Found, %
178–180 (decompn.)	82.47	88.57	17.38	11.43	99.85
183–184 (decompn.)	84.51	88.57	15.17	11.43	99.68
187–188 (decompn.)	86.63	88.57	12.59	11.43	99.22
189–191 (decompn.)	89.03	88.57	10.49	11.43	99.52

[a] All values are calculated on dried substance (100° *in vacuo*). The present data are average values of three parallel analyses.

assay of tartaric acid in ergotamine tartrate was carried out by a modified titrimetric method of Gyenes and Szász (5), using 0.1 *N* potassium methoxide and naphtholbenzein indicator which gave an exact end point.

All samples were checked for purity by paper chromatograms using a modified Macek method (R_f of ergotamine, about 0.1) (4, 6, 7). No ergotaminine or other alkaloid contaminant was found.

DISCUSSION

As is shown in Table II, ergotamine tartrate U.S.P. contains about 60% ergotamine bitartrate and 40% ergotamine tartrate. On the basis of the found base content, calculated on ergotamine tartrate, there is a deviation of about 6% (in case of ergotamine bitartrate, 10.25%) from 100.00% ergotamine tartrate.

The van Urk colorimetric method (*p*-dimethylaminobenzaldehyde T.S. as reagent) yields an accuracy of about ±4–6% when the U.S.P. XVI directions are followed. Positive errors of estimation reduce the deviation partly or completely, recording a more-or-less pure ergotamine tartrate. Against that, negative errors in the van Urk method and the alleged error in composition (due to presence of bitartrate) together caused a deviation of up to 10 to 12%. Such a variation can be avoided by application of the much more exact perchloric acid method. Furthermore, the use of an exact method is not sufficient to reach the minimal U.S.P. requirement of 97.0%, simultaneously maintaining the requirement for melting point of 180° (decompn.) (see Tables II and III); these two requirements are incompatible.

A good soluble ergotamine salt, *e.g.*, ergotamine bitartrate, is often requested for solutions or injections. In our opinion, the slighter solubility of ergotamine tartrate is not disadvantageous for the use in pharmaceutical preparations, because in the case of tablets, ergotamine (base or tartrate) is absorbed by the stomach in the form of the hydrochloric salt, and in the case of injections or solutions (prescribed pH 3–4, adjusted by tartaric acid), ergotamine tartrate is converted into the bitartrate salt.

SUMMARY

1. Quantitative studies using titrimetric methods in nonaqueous media indicated that ergotamine tartrate U.S.P. with a melting point of 180° (decompn.) contains about 60% ergotamine bitartrate.

2. Melting points between 170–192° (decompn.) indicate the approximate ratio of ergotamine bitartrate-tartrate mixture.

3. Application of the perchloric acid titrimetric method (accuracy, ±0.5%) for the assay of ergotamine tartrate substance yields more exact quantitative data than the official van Urk colorimetric method (accuracy, ±4–6%).

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Notes

Quaternary Ammonium Germicides as Surface Disinfectants

By CHARLES J. BANKS†, GEORGE F. REDDISH, and C. LEE HUYCK‡

The quaternary ammonium compounds tested proved to be excellent germicides in the dilutions recommended by the manufacturer for waxed and unwaxed floor coverings. In the case of waxed stainless steel, it was assumed that the wax interfered with the disinfectant action because the unwaxed stainless steel was found to be sterile. Quaternary ammonium germicides were found to be unsatisfactory disinfectants for vinyl tile.

THE PURPOSE of this study is to determine whether the quaternary ammonium germicides are useful as sanitizing agents in preventing the spread of infections caused by the staphylococci organisms.

The quaternary ammonium germicides are odorless, colorless, highly stable, and are relatively nontoxic when used in their recommended germicidal concentrations. In general, the quaternary ammonium germicides have been found to inhibit the oxidation of certain carbohydrates (1) and thereby interfere with respiration and glycolysis of bacteria. They are usually tested by the phenol coefficient method. Since in the United States 5% phenol is accepted as the standard of excellence for general disinfection, Reddish (2) has pointed out that dilutions 20 times the phenol coefficient afford a wide margin of safety. The dilution calculated on this basis is usually of sufficient strength to kill all pathogenic microorganisms which cause epidemics.

Although the phenol coefficient is an excellent *in vitro* test, in light of the increase of staphylococcus infections in hospitals, a practical test is recommended. The phenol coefficient test employs *Salmonella typhosa* which is similar in resistance to most disease-producing microorganisms. There are some organisms that are more resistant to disinfectants than *Salmonella typhosa*, one of which is the nonspore forming *Staphylococcus aureus*.

Of particular importance to this study are the incompatabilities of the quaternary ammonium germicides which occur under normal sanitizing conditions. Domagk (3) in 1935 was the first to call attention to the interfering action of ordinary soap on the quaternary ammonium germicides. Since then, incompatibilities such as the nitrate and benzalkonium chloride incompatibility (4) have been pointed out. It is generally recognized that when surfaces have been washed with ordinary soap and water, the objects must be thoroughly rinsed with water before applying a quaternary ammonium disinfectant for sterilizing purposes. This applies to the disinfection of wounds following the cleansing procedure with soap and water.

EXPERIMENTAL[1]

Roccal 10%[2]—The active ingredient is a mixture of technical grade alkyldimethylbenzyl ammonium chlorides in which the alkyl is a mixture of C_8H_{17} to $C_{18}H_{37}$ groups. The phenol coefficient of Roccal 10% is 25 when tested against *Salmonella typhosa* at 20°. It is used for sanitizing eating and drinking utensils.

Quaternary Ammonium Germicide.—The active

Received December 11, 1961, from the St. Louis College of Pharmacy, St. Louis, Mo.

Accepted for publication January 23, 1962.

The senior author acknowledges the constant advice and assistance given him by Sister Mary Quentin, St. Mary's Hospital, St. Louis, Mo.

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[1] Tabulated data of the experimental results are available from Dr. C. Lee Huyck, Division of Eaton Laboratories of the Norwich Pharmacal Co., Norwich, N. Y.

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ingredient is *p*-diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride. The phenol coefficient is 20. It is used in sanitizing sickrooms.

Anti-Staph. Concentrated Antiseptic Cleaner.—The active ingredients are 10.75% methyliodododecylbenzyltrimethyl ammonium chloride, isopropyl alcohol, methylododecyl xylylene bis-(trimethyl ammonium chloride), triethanolamine, potassium hydroxide, and tetrasodium salt of ethylenediamine tetraacetic acid. Inert ingredients include water, nonyl phenoxypolyoxyethylene ethanol, perfume oils, artificial color, and citric acid. The phenol coefficient by a modified Rideal-Walker method against *Salmonella typhosa* is 10 and against *Staphylococcus aureus*, 20. It is used for ordinary cleansing, sanitizing, and deodorizing.

Braxene.[3] The active ingredients are 20% high molecular weight alkyl (C_{12}, C_{14}, C_{16}, and related C_8 to C_{18}) dimethylbenzyl ammonium chlorides. Water (less than 25 p.p.m. hardness) is the diluent. Braxene is used in the asepsis of linen. The phenol coefficient for *Salmonella typhosa* is 85 at 37° and 50 at 20°; the phenol coefficient for *M. pyogenes* var. *aureus* is 81 at 37° and 55 at 20°. In addition to its effectiveness in the treatment of linen, Braxene is also recommended as a disinfectant for sorting tables, storage shelves, laundry carts, and other equipment used in the handling and storage of clothing.

Blue Chip Concentrate.[4]—The active ingredients are *n*-alkyl (50% C_{12}, 30% C_{14}, 17% C_{16}, 3% C_{18}) dimethylbenzyl ammonium chlorides 1.25%. Inert ingredients are water, detergents, a synergizing compound, water softener, dye, and perfume. In addition to cleaning and disinfecting surfaces it deodorizes, and sanitizes.

METHOD

In any practical test, a large number of organisms must be used. Floor materials used in this test were: painted wood, vinyl tile, terrazzo, and linoleum. Stainless steel was also used because of its widespread use in hospitals.

Unwaxed and waxed floor coverings were used in the tests. After washing with soap and water and rinsing with plain tap water, the slabs of floor coverings (9 in. × 9 in.) were air dried for 20 minutes. The slab was divided into 5 equal parts designated: control I, control II, blank, 5 minutes, 15 minutes, and 30 minutes. One milliliter of 1:1000 dilution of a 24-hour broth culture of *Staphylococcus aureus* at 37° was uniformly spread over the surface of the slab by means of a sterile pipet and allowed to air dry for 20 min. A control count was made on control I by streaking it 3 times with a sterile cotton swab moistened with sterile water followed by inoculation into 20 ml. of nutrient agar, swirled vigorously for 15 sec., poured into a sterile Petri dish, incubated for 48 hours at 37°. Immediately after the control swabs were made, portions designated as 5, 15, and 30 min. were covered with 2 ml. of the dilution of solution being tested which was either 20 times the phenol coefficient, or the dilution (5) recommended by the manufacturer. At the end of 5, 15, and 30 min., the portion of the slab so designated was streaked 3 times with a sterile cotton swab moistened with sterile water, followed by inoculation into 20 ml. of nutrient agar, and incubated at 37° for 48 hours. At the end of the 30-min. period, another control swabbing was made from the second control area designated control II, inoculated as previously into nutrient agar, and incubated. The total counts of the staphylococci present were made and the reductions from the control counts determined.

[3] Wyandotte Chemicals Corp., Wyandotte, Mich.
[4] S. C. Johnson & Son, Inc., Racine, Wis.

DISCUSSION

The floor coverings used were either porous or nonporous. The porous floors were either painted wood or terrazzo, while nonporous were vinyl tile or linoleum. The porous material gave a lower count than the nonporous for the controls, but this material had no effect on the germicidal activity of the quaternary germicides. In the test, a portion was designated as blank between control II and a portion designated as 5 min. These designations were safety measures to prevent the germicides from reaching the control area. Each plate that exhibited no growth was streaked with a 24-hr. culture of the test organism and incubated at 37° for 48 hours. Growth occurred in each instance, showing no bacteriostatic action of the plates. In swabbing the floor coverings, the germicide could be transferred from the floor covering to the agar. If a substantial amount was transferred, the plate would exhibit bacteriostatic action.

Phenol 5% and cresol compound 2% were used as standards for comparison since they are excellent germicides under conditions of use. Therefore, all germicides used in these tests should have the same relative bactericidal efficacy.

SUMMARY

Phenol 5% and cresol compound 2% were used as standards in performance tests on various floor coverings. The quaternary ammonium germicides were compared with these standard disinfectants on waxed and unwaxed floor coverings. The quaternary ammonium compounds tested proved to be excellent germicides in the dilutions recommended by the manufacturer. Wax on the floor coverings did not interfere with germicidal action. In the case of stainless steel, wax did interfere with the disinfectant action, probably because the wax was not absorbed on the metal. Only in one case did an organic material interfere with the germicidal activity of the quaternary ammonium compound; the interfering substance in this case was unwaxed vinyl tile.

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Determination of *m*-Aminophenol (MAP) in Calcium Aminosalicylate [$Ca(PAS)_2$]

By R. B. LUERS, Jr., and L. B. STADLER

The MAP test in the N.F. XI monograph for $Ca(PAS)_2$ has been re-evaluated. Experimental data showed the test to be in need of minor changes to correct sources of error. Based on this study, recommendations are made to use sulfuric acid instead of hydrochloric acid, to change the correction factor, to determine maximum absorbance through a range of 425 to 435 mμ, and to change the time allowed for color development to 3 hours.

THE MAP test in the N.F. XI monograph for $Ca(PAS)_2$ (1) is basically a good analytical method. However, our previous work with aminosalicylic acid (PAS) and sodium aminosalicylate (PASNa) (2) showed that certain discrepancies existed in the MAP test. Subsequent studies made on $Ca(PAS)_2$ showed that the variables affecting the MAP test for PAS and PASNa applied also to the MAP test for $Ca(PAS)_2$.

To establish the absolute MAP content of some production samples of $Ca(PAS)_2$, the chromatographic procedure used in our previous studies of PAS and PASNa (2) was applied (see Table I).

EXPERIMENTAL

Proposed Modified Colorimetric Method for MAP in $Ca(PAS)_2$.—The proposed revisions of the N.F. XI test for MAP, as described under *Procedure*, were performed on samples of $Ca(PAS)_2$. The N.F. XI test for MAP was performed also on the same samples of $Ca(PAS)_2$. The basis for the revisions proposed are discussed under *Conditions Affecting the Colorimetric Determination of MAP.*

Conditions Affecting the Colorimetric Determination of MAP

Effect of Acid Used upon Absorbance Maximum of the Color Developed.—Samples of $Ca(PAS)_2$ tested by the N.F. XI method, using hydrochloric acid, gave absorbance values at 440 mμ that were about 3% lower than absorbance values obtained using sulfuric acid with the N.F. XI procedure (see Table II). In the proposed modified method described under *Procedure*, the absorbance values using hydrochloric acid were about 3 to 5% lower than were the absorbance values using sulfuric acid. Also, it was found that the use of hydrochloric acid in the initial solution sometimes produced a precipitate which could complicate the test. Based on these results and to be consistent with the proposed revised colorimetric procedure recommended for MAP in PAS and PASNa in our previous studies (2), sulfuric acid was selected.

Effect of Time upon the Color Intensity Developed.—As had been established by our previous investigations (2), the color intensity stabilized in about 3 hours after the addition of the sodium carbonate solution. Since our studies showed this to occur in the MAP test for $Ca(PAS)_2$ as well, the time lapse between the addition of the sodium carbonate solution and the measurement of color intensity has been changed to 3 hours in the proposed revised colorimetric method for MAP in $Ca(PAS)_2$, as described under *Procedure*. This characteristic in the color development was found evident in all samples of $Ca(PAS)_2$ tested.

Effect of Change in Concentration of MAP upon the Absorbance Maximum of the Color Developed.—As was shown in our previous studies (2), a shift in absorbance maximum occurred as the concentration of MAP in $Ca(PAS)_2$ was varied from 0.02 to 1.0% (see Table III). These results justified the use of a 425 to 435 mμ range for the measurement of absorbance of the color developed by the proposed revised colorimetric method described under *Procedure* for the determination of MAP in $Ca(PAS)_2$. None of the samples of $Ca(PAS)_2$ tested showed absorbance maxima at 440 mμ.

Absorbance Correction Value for Color Produced Other Than by MAP.—Based upon our study of a number of production samples of $Ca(PAS)_2$ and the correlation of data between the paper chromatographic procedure and the proposed modified colorimetric method for MAP, it was clearly established that the correction value for color produced other than by MAP should be 0.320 rather than 0.372 (see Table I).

Procedure

Weigh accurately an amount, calculated on the basis of the *Assay*, of Calcium Aminosalicylate equivalent to 562 mg. of anhydrous calcium aminosalicylate (500 mg. of aminosalicylic acid), and transfer to a 100-ml. volumetric flask. Add 1.8 ml. of sodium hydroxide T.S., and dilute to about 80 ml. with water. Add 10 ml. of dilute sulfuric acid (1 in 10), add water to volume, and mix. Within 150 seconds from the time the acid is added, transfer 5 ml. of this

TABLE I.—PER CENT OF MAP FOUND IN $Ca(PAS)_2$ BY THE DIFFERENT METHODS TESTED

Sample of $Ca(PAS)_2$ Tested	MAP Found, %		
	By Paper Chromatography	By N.F. XI Method	By Proposed Modified Method
I	<0.01	negligible[a]	<0.01
II	<0.01	negligible	<0.01
III	<0.01	negligible	<0.01
IV[b]	...	0.04	0.14
V[b]	...	0.05	0.17
VI[b]	0.11	negligible	0.11
VII	...	negligible	0.03
VIII	...	negligible	0.03
IX	<0.01	negligible	<0.01
X	...	negligible	<0.01
XI	...	negligible	0.01
XII	...	negligible	<0.01

[a] The term "negligible" denotes that the absorbance value for the sample was <0.372, which by the N.F. XI method would result in a minus percentage value for MAP content. [b] Experimental laboratory samples stored for 8 years.

Received November 1, 1961, from the Analytical Laboratories, Parke, Davis & Co., Detroit 32, Mich.
Accepted for publication January 29, 1962.
The authors are greatly indebted to Mr. F. A. Maurina for guidance and technical assistance given. The authors wish to express their gratitude to Mr. Carl E. Greco for paper chromatography and to Mr. Richard C. Zingle for spectrophotometric curves. Samples were kindly supplied by Miss Phyllis Rhodes, Dorsey Laboratories, Division of the Wander Co., Lincoln 1, Nebr.

TABLE II.—EFFECT OF ACID USED UPON THE ABSORBANCE MAXIMUM OF THE COLOR DEVELOPED

Sample	N.F. XI Method Absorbance Using HCl	N.F. XI Method Absorbance Using H_2SO_4	Modified Method Absorbance Using HCl	Modified Method Absorbance Using H_2SO_4
I	0.315	0.324	0.312 (435 mμ)	0.321 (425 mμ)
II	0.310	0.320	0.305 (430 mμ)	0.320 (425 mμ)
III	0.305	0.315	0.307 (430 mμ)	0.322 (425 mμ)

TABLE III.—EFFECT OF CHANGE IN CONCENTRATION OF MAP UPON ABSORBANCE MAXIMUM

MAP in $Ca(PAS)_2$, %	Absorbance Maximum Found, mμ	Comment on Curve Characteristics
0.02	425	Sloping plateau occurs
0.05	425	Plateau occurs
0.10	430	Round curve occurs
0.20	430–435	Round curve occurs
0.50	435	Sharp peak occurs
1.00	435	Sharp peak occurs

solution to a second 100-ml. volumetric flask, immersed in an ice bath and containing 50 ml. of water at 0 to 5°, and add 2.5 ml. of sodium nitrite solution (1 in 100). Mix and allow to stand in the ice bath for 3 minutes ± 5 seconds. Add 25 ml. of sodium carbonate T.S., mix, and place the flask in a water bath at 25° for 15 minutes. Add water to volume, mix, allow the solution to stand at 25° for 3 hours, and filter. Determine the absorbance of the clear solution at the absorbance maximum observed in a range of 425 to 435 mμ in a 1-cm. cell with a suitable spectrophotometer, using water as the blank. Calculate the per cent of *m*-aminophenol by the formula $(A - 0.320/1.09)$, in which A is the absorbance of the solution, 0.320 is the absorbance correction factor representing color produced other than by the reaction of *m*-aminophenol initially present, and 1.09 is the factor converting absorbance to per cent of *m*-aminophenol in Calcium Aminosalicylate.

SUMMARY AND CONCLUSIONS

1. The MAP content found by the proposed modified colorimetric method was comparable to that found by paper chromatography for the $Ca(PAS)_2$ tested in this study.

2. In the addition of acid to the initial solution in the MAP test, sulfuric acid should be added instead of hydrochloric acid.

3. The color produced by both the N.F. XI and the proposed modified colorimetric method stabilized after standing for 3 hours at 25°.

4. The absorbance spectra obtained for MAP by both the N.F. XI method and the proposed modified colorimetric method showed the absorbance maxima to be between 425 and 435 mμ.

5. The correction factor for color produced other than by MAP should be 0.320, not 0.372.

6. The N.F. XI method for the determination of MAP in $Ca(PAS)_2$ is a basically reliable procedure.

7. The value, 1.09, for the conversion of absorbance to per cent MAP was correct.

8. It is recommended that the modifications, as herein described, be adopted by N.F. XI to correct minor sources of error in the determination of MAP in $Ca(PAS)_2$.

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Ultraviolet Emission Spectrographic Determination of Trace Palladium to Ascertain the Method Used to Produce Tetracycline

By WILLIAM L. DUTTON and R. C. HIRT

Ultraviolet emission spectrography may be readily employed to determine trace amounts of palladium in ashed samples of tetracycline. Palladium is not found in samples prepared by direct fermentation and is always found when chlortetracycline is dechlorinated using palladium catalytically.

TWO KNOWN commercial methods are used to produce the antibiotic tetracycline. The catalytic reduction of chlortetracycline using palladium or another of the noble metals (1) and the direct fermentation of the microorganism *Streptomyces aureofaciens* (2).

METHOD

The general procedure is essentially the carbon arc method of qualitative analysis by Harvey (3) and Nachtrieb (4). The sensitivity of the method was increased by using the slowly rotating slotted disk and stationary emulsion method (5). Using a 35-mg. charge, 0.1 mcg. or 3 p.p.m. can be detected. A charge containing 3.5 mcg. or 100 p.p.m. of palladium produces up to 10 spectral lines of the metal in the region 3500 to 2450 Å.

The 600–800° ash cake is ground fine in the porcelain crucible. The exposure is 3 min. as required by the 11-slot disk (5). The spectral line palladium 3242.703 Å. is used for detecting the least amount of palladium, even though it is theoretically not the strongest. It is conveniently close to copper 3247.540 Å. for positive identification and has very little interference from a carbon line.

The length, width, and density of the line are mentally integrated into an order of magnitude value (3, 4) and applied to the least detectable concentration of 3 p.p.m. The least detectable concentration is different in each ashed sample. It depends greatly upon the amount of reduction in weight by dry ashing at 600 to 800°.

Received June 3, 1961, from American Cyanamid Co., Central Research Division, Stamford, Conn.
Accepted for publication February 26, 1962.

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TABLE II.—EFFECT OF ACID USED UPON THE ABSORBANCE MAXIMUM OF THE COLOR DEVELOPED

Sample	N.F. XI Method Absorbance Using HCl	N.F. XI Method Absorbance Using H_2SO_4	Modified Method Absorbance Using HCl	Modified Method Absorbance Using H_2SO_4
I	0.315	0.324	0.312 (435 mμ)	0.321 (425 mμ)
II	0.310	0.320	0.305 (430 mμ)	0.320 (425 mμ)
III	0.305	0.315	0.307 (430 mμ)	0.322 (425 mμ)

TABLE III.—EFFECT OF CHANGE IN CONCENTRATION OF MAP UPON ABSORBANCE MAXIMUM

MAP in $Ca(PAS)_2$, %	Absorbance Maximum Found, mμ	Comment on Curve Characteristics
0.02	425	Sloping plateau occurs
0.05	425	Plateau occurs
0.10	430	Round curve occurs
0.20	430–435	Round curve occurs
0.50	435	Sharp peak occurs
1.00	435	Sharp peak occurs

solution to a second 100-ml. volumetric flask, immersed in an ice bath and containing 50 ml. of water at 0 to 5°, and add 2.5 ml. of sodium nitrite solution (1 in 100). Mix and allow to stand in the ice bath for 3 minutes ± 5 seconds. Add 25 ml. of sodium carbonate T.S., mix, and place the flask in a water bath at 25° for 15 minutes. Add water to volume, mix, allow the solution to stand at 25° for 3 hours, and filter. Determine the absorbance of the clear solution at the absorbance maximum observed in a range of 425 to 435 mμ in a 1-cm. cell with a suitable spectrophotometer, using water as the blank. Calculate the per cent of *m*-aminophenol by the formula $(A - 0.320/1.09)$, in which A is the absorbance of the solution, 0.320 is the absorbance correction factor representing color produced other than by the reaction of *m*-aminophenol initially present, and 1.09 is the factor converting absorbance to per cent of *m*-aminophenol in Calcium Aminosalicylate.

SUMMARY AND CONCLUSIONS

1. The MAP content found by the proposed modified colorimetric method was comparable to that found by paper chromatography for the $Ca(PAS)_2$ tested in this study.

2. In the addition of acid to the initial solution in the MAP test, sulfuric acid should be added instead of hydrochloric acid.

3. The color produced by both the N.F. XI and the proposed modified colorimetric method stabilized after standing for 3 hours at 25°.

4. The absorbance spectra obtained for MAP by both the N.F. XI method and the proposed modified colorimetric method showed the absorbance maxima to be between 425 and 435 mμ.

5. The correction factor for color produced other than by MAP should be 0.320, not 0.372.

6. The N.F. XI method for the determination of MAP in $Ca(PAS)_2$ is a basically reliable procedure.

7. The value, 1.09, for the conversion of absorbance to per cent MAP was correct.

8. It is recommended that the modifications, as herein described, be adopted by N.F. XI to correct minor sources of error in the determination of MAP in $Ca(PAS)_2$.

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Ultraviolet Emission Spectrographic Determination of Trace Palladium to Ascertain the Method Used to Produce Tetracycline

By WILLIAM L. DUTTON and R. C. HIRT

Ultraviolet emission spectrography may be readily employed to determine trace amounts of palladium in ashed samples of tetracycline. Palladium is not found in samples prepared by direct fermentation and is always found when chlortetracycline is dechlorinated using palladium catalytically.

TWO KNOWN commercial methods are used to produce the antibiotic tetracycline. The catalytic reduction of chlortetracycline using palladium or another of the noble metals (1) and the direct fermentation of the microorganism *Streptomyces aureofaciens* (2).

METHOD

The general procedure is essentially the carbon arc method of qualitative analysis by Harvey (3) and Nachtrieb (4). The sensitivity of the method was increased by using the slowly rotating slotted disk and stationary emulsion method (5). Using a 35-mg. charge, 0.1 mcg. or 3 p.p.m. can be detected. A charge containing 3.5 mcg. or 100 p.p.m. of palladium produces up to 10 spectral lines of the metal in the region 3500 to 2450 Å.

The 600–800° ash cake is ground fine in the porcelain crucible. The exposure is 3 min. as required by the 11-slot disk (5). The spectral line palladium 3242.703 Å. is used for detecting the least amount of palladium, even though it is theoretically not the strongest. It is conveniently close to copper 3247.540 Å. for positive identification and has very little interference from a carbon line.

The length, width, and density of the line are mentally integrated into an order of magnitude value (3, 4) and applied to the least detectable concentration of 3 p.p.m. The least detectable concentration is different in each ashed sample. It depends greatly upon the amount of reduction in weight by dry ashing at 600 to 800°.

Received June 3, 1961, from American Cyanamid Co., Central Research Division, Stamford, Conn.
Accepted for publication February 26, 1962.

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Relative Antiplasmin (Antifibrinolysin) Activity of ω-Aminocarboxylic Acids

By MICHIO MIYAMOTO† and KWAN-HUA LEE

ω-Aminocarboxylic acids with carbon chain lengths of three to eight are shown to possess antifibrinolytic activity *in vitro*. ω-Aminohexanoic acid (ε-aminocaproic acid) is the most active compound studied.

IN RECENT YEARS one of the most exciting achievements in therapeutics probably has been the introduction of several enzymes for clinical uses. One of the latest developments is human plasmin (or fibrinolysin) preparation. Plasmin is the enzyme normally present in the blood plasma in its inactive zymogen form, known as plasminogen. In the laboratory, plasminogen may be converted into plasmin by streptokinase or urokinase. Plasmin is used in the treatment of venous thrombosis, such as thrombophlebitis or phlebothrombosis, pulmonary embolism, and topical debridements. However, overdosage of injected plasmin may cause hypofibrinogenemia and uncontrolled hemorrhage. An antiplasmin agent which serves as an antidote in controlling plasma plasmin activity is highly in need.

In 1957 workers in Japan announced the discovery of an antiplasmin (antifibrinolysin) agent known as ε-aminocaproic acid. This compound was patented in Great Britain (1) and distributed by Daiichi Seiyaku Co. of Japan. Remarkable effects were obtained by the Japanese workers on cases of hemorrhagic metropathia, dysmenorrhea, threatened abortion, toxemia of pregnancy, hyperemesis gravidarum, and eczema urticaria and other dermatitis. All of these diseases are thought to be associated with increased plasmin activity. They gave this acid in doses of 10–20 Gm. per day, orally or intravenously, to more than 100 patients and observed no toxic effect (2).

The effect of ε-aminocaproic acid on a large number of clinical coagulation defects was systemically investigated in Sweden in 1960 (3). Enhanced fibrinolysis has been reported in various diseases. Pathological fibrinolysis most probably arises from increased circulating levels of plasminogen activator. Fibrinogen, often given in large quantities under these circumstances, is usually without significant effect because of its rapid destruction by plasmin. Theoretically, control of fibrinolysis by giving an inhibitor of plasminogen activation, followed by the use of fibrinogen, should be satisfactory. In these respects, ε-aminocaproic acid has proved to be promising. Favorable results have been obtained by Nilsson, Sjoerdsma, and Waldenstrom (3) in a limited number of clinical cases.

It has been suspected for some time that activated blood fibrinolysin may also play a prominent role in the pathogenesis of anaphylactic shock. Recently, Burden and co-workers (4) have demonstrated that the spontaneous lysis of clotted blood samples collected during acutely fatal shock in guinea pigs following intravenous injection of peptone, histamine, trypsin, and anaphylatoxin was found to be a regular feature of these reactions just as observed previously in guinea pigs undergoing specific anaphylactic shock (5). With the aid of a potent fibrinolysin inhibitor, it is possible to find out whether increased fibrinolytic activity is the consequence or the cause of anaphylactic shock.

The potential importance of this antiplasmin agent prompted our interest in studying the mode of action of ε-aminocaproic acid. As the first step of approach, we chose to study the relative antiplasmin activity of the homologous series of ω-aminocarboxylic acids. The results of this study are reported in this paper.

METHODS AND MATERIALS

Measurement of Fibrinolytic Activity.—For the measurement of fibrinolytic activity, the method described by Lewis and Ferguson was used with modifications (6). To a small tube add 0.5 ml. of 1% human plasma fraction I (fibrinogen) and 0.5 ml. of borate buffer (0.1*M*, pH 7.5). To a second tube, add 0.5 ml. of enzyme solution (purified human plasmin solution, 150 units) and 0.5 ml. of human thrombin (2 units). Mix the contents of the tubes by pouring back and forth twice, and then incubate at 37°. The amount of fibrinogen in the tube after being acted upon by the excess amount of thrombin present was just enough to form a turbid gelatinous fibrin solution. The amount of plasmin added was sufficient to clarify the turbid solution about 10 minutes after incubation. Buffer alone or buffer with antiplasmin agents was added in a volume of 0.3 ml. to the fibrinogen solution. The final volume in all cases was 2.3 ml. Time for the clarification of the gelatinous fibrin solution was recorded. The effect of concentration of different ω-amino acids was assayed separately at first, since preliminary experimental results indicated that the concentration range varied widely. The effective concentration range for each acid studied has been located with this method. The purpose of this type of study was to supply information for selecting an appropriate concentration at which the activities of all of the acids studied could be compared. Under the conditions described in this paper, the concentration selected was 50 mmoles per L., and the relative antifibrinolysin activity of ω-aminocarboxylic acids at this concentration was then studied.

Materials Used.—The biological materials used in the present studies were all commercial products.

Received December 9, 1961, from the University of California, School of Pharmacy, San Francisco 22.

Accepted for publication January 12, 1962.

The authors wish to thank Dr. Joseph Krezanoski of Barnes-Hind Laboratories, Sunnyvale, Calif., who first called our attention to the biological activity of ε-aminocaproic acid, for his untiring interest in this work.

† Recipient of the Lunsford Richardson Pharmacy Award, 1961.

Human fibrinogen used was Parenogen of Cutter Laboratories and the fibrinolysin used was Actase of Ortho Pharmaceutical Corp. Thrombin was obtained from Upjohn Co. All of the ω-aminocarboxylic acids used were commercial preparations and were recrystallized from either water or ethanol four times and dried in a vacuum desiccator over phosphorus pentoxide.

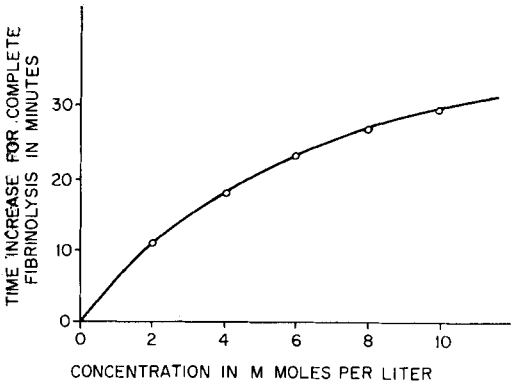


Fig. 1.—Antiplasmin activity of ε-aminocaproic acid.

RESULTS AND DISCUSSION

The inhibitory effect on fibrinolysis of each of the ω-aminocarboxylic acids at graded concentrations was studied. Only the results of the study on ε-aminocaproic acid are presented in Fig. 1 as representative of these studies. The relative antifibrinolysin activity of the ω-amino acids at 50 mmoles per L. concentration was studied in four experiments and the results obtained, in general, were essentially the same. Representative results of these studies are shown in Fig. 2. The results indicated that ε-aminocaproic acid was the most active compound. The shape of the curve in Fig. 2 suggests a sharp, geometrical fitness of the ε-aminocaproic acid molecule at the site of inhibition. The natural amino acid lysine is α,ε-diaminocaproic acid. It is a known fact that plasmin, like trypsin, can specifically hydrolyze the lysinyl bond of lysinyl peptides or lysinyl esters. These facts raised the question as to whether or not ε-aminocaproic acid exerts its inhibitory action by competing with the lysine moiety in the fibrin molecule. It was unexpected that L-lysine did not inhibit plasmin activity even at higher concentrations. At very high concentration of lysine solution,

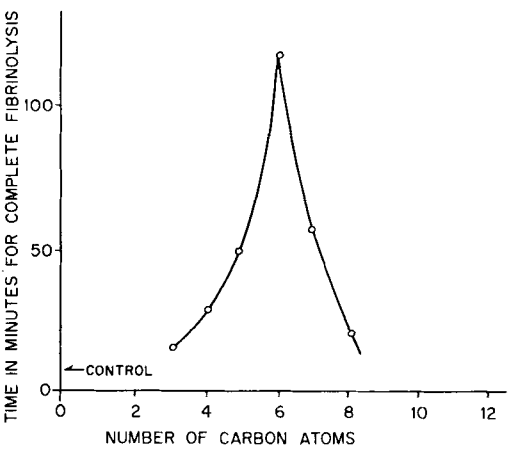


Fig. 2.—Relative antiplasmin activity of ω-aminocarboxylic acids at 50 mmoles per L.

fibrin was soluble. D-Lysine or L-arginine showed no inhibitory effect. We also found that lysine exerted no effect on the antiplasmin effect of ε-aminocaproic acid. It is interesting to point out that γ-aminobutyric acid also has considerable antiplasmin activity. This finding may be of significance since no other clue has been found on the physiological or biochemical function or functions of the large amount of γ-aminobutyric acid generally present in the normal central nervous system.

The low toxicity (3, 7) and high activity of ε-aminocaproic acid are the desirable properties of a useful therapeutic agent; however, its rapid rate of excretion (3) reduces its practical use. Further studies on these problems are under way.

SUMMARY

An enzyme system for assaying antifibrinolytic activity is described. The relative antifibrinolysin activity of the homologous series of ω-aminocarboxylic acids has been studied. It has been found that ε-aminocaproic acid has the highest antifibrinolytic activity.

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

October 1962 volume 51, number 10

Review Article

Absorption of Implanted Solid Drug

By BERTON E. BALLARD and EINO NELSON†

DRUG ABSORPTION is a subject which is attracting increasing interest in the areas of pharmacy and pharmacology. The technique of solid drug pellet implantation has particular importance in the livestock and poultry fields, in the area of cancer research where carcinogens or potential ones are studied, in theoretical studies involved with solid drug absorption, in endocrinological work, in studies concerned with metabolism and fate of drugs, and in many more areas where a prolonged "continuous infusion" of drug is required.

Recent reviews have summarized much of what is known concerning processes and factors influencing rate of absorption when drugs are administered by several routes. Thus, with respect to gastrointestinal absorption, Schanker (1) has discussed mainly the influence of a drug's dissociation constant and fat solubility on the process, and Wagner (2) has reviewed the influence of solution kinetics. Cooper and Lazarus (3, 4) and Nelson (5) have considered mainly the properties of sustained-release dosage forms intended for oral administration. Absorption following subcutaneous injection has been exhaustively discussed by Schou (6). The paper of Riegelman and Crowell (7) reviews information available on absorption after administration of drug by the rectal route. Past work on percutaneous absorption has been summarized by Gemmel and Morrison (8) and Barr (9). Work on sublingual absorption has been listed by Katz and Barr (10).

Apparently there has been no general review on absorption after implantation of either pure solid drug or solid drug in mixture with either inert or active materials, although brief reviews on specific topics have appeared. These are listed in an Appendix.[1] This review has been prepared in two parts. In the first part are considered the various physiological and physical factors that have been found to influence absorption rate. The second part of the review is presented in the form of an Appendix and lists and gives references to work in which a variety of drugs have been implanted. For simplicity in organization, each part of the review has its own set of references, numbered consecutively. One reason for the organization adopted is that there is much work reported in the literature which merely describes pharmacological effects elicited from the implanted drugs and bears only indirectly on absorption *per se*. This type of work is listed in the Appendix. Also listed in the Appendix are more complete references to some of the subjects discussed in the main body of the review itself.

In some ways, understanding absorption of implanted solid drug is simplified by the physical nature of materials logically administered by this route. Implantation is nearly synonymous with

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[1] EDITOR'S NOTE: The Appendix which is referred to is not published with this review article in THIS JOURNAL. However, copies of it, in mimeographed form, are available on request from the authors, and interested readers should send their written request direct to the authors.

long duration of action and, as a consequence of this, recent work has shown that understanding the absorption process in many cases is reduced to the problem of understanding solution kinetics *in vivo* (11), because release of drug from pellet implant in the usual case is a much slower process than passage of drug across the membrane at the absorption site. Absorption, then, is solution rate limited.

Solution Kinetics.—A number of factors influence the rate of the solution *in vitro* and *in vivo*. Most of these factors have been discussed in detail in recent reviews (2, 11, 12). One of the most important aspects of absorption from implant is the influence of the implant's geometry and how its weight and surface area change with time.

Influence of the Geometry and/or Surface Area of Implant on Absorption.—Apparently Shelesnyak and Engle (13) were the first to recognize that absorption rate was proportional to the surface area of the implant at a given time. This finding has been confirmed since then, without exception, in many investigations. Surface area will decrease with time, and the function describing the rate of decrease is given by the derivative of the expression describing surface area as a function of time with respect to time.

For regular geometric objects, surface area is usually easy to express as a function of the dimensions of the objects. Thus, the surface area, S, of a sphere is given by

$$S = \pi D^2 \qquad \text{(Eq. 1)}$$

If diameter, D, decreases uniformly with time, *cf.* (14–20), then

$$dD/dt = -k \qquad \text{(Eq. 2)}$$

where k is the rate of decrease of diameter with time. On integrating Eq. 2 and evaluating the constant of integration at zero time, the following equation results

$$D = D^0 - kt \qquad \text{(Eq. 3)}$$

where D^0 is the initial diameter. Substituting Eq. 3 into 1 gives

$$S = \pi (D^0 - kt)^2 \qquad \text{(Eq. 4)}$$

as the relationship between surface area and time. The weight, W, of spheres is given by

$$W = \frac{\pi\rho}{6} D^3 \qquad \text{(Eq. 5)}$$

where ρ is the density of the drug. Decrease of weight with time will be given, using Eq. 3

$$W = \frac{\pi\rho}{6} (D^0 - kt)^3 \qquad \text{(Eq. 6)}$$

or by substituting for D^0 in Eq. 6 by its value from Eq. 5

$$W = \frac{\pi\rho}{6} \left[\left(\frac{6\,W^0}{\pi\rho} \right)^{1/3} - kt \right]^3 \qquad \text{(Eq. 7)}$$

Equation 7 solved for k becomes

$$k = \frac{1}{t} \left[\left(\frac{6\,W^0}{\pi\rho} \right)^{1/3} - \left(\frac{6W}{\pi\rho} \right)^{1/3} \right] \qquad \text{(Eq. 8)}$$

Alternatively, the value of k could be found by dividing the difference between initial and final diameter by the length of time in implant. However, in general, weight is measured more accurately than dimensions because of slight surface irregularities and shape distortions. Once k is determined from experimental measurements, it may be substituted back into Eq. 6 or 7, and weight may then be calculated at any time. Further, when the derivative of Eq. 6 is taken, instantaneous absorption rate, dA/dt, with negative sign is given. Thus

$$\frac{dW}{dt} = -\frac{dA}{dt} = -\frac{k\pi\rho}{2} (D^0 - kt)^2 \qquad \text{(Eq. 9)}$$

Data on the absorption of tolbutamide from implanted spheres of this material, as given by Ballard and Nelson (21), serves as an example to illustrate information that can be obtained from some of the preceding equations. Using their data for sphere B (*loc. cit.*), Eq. 6 becomes

$$W = 606\,(1.048 - 0.00245t)^3 \qquad \text{(Eq. 10)}$$

where W is in mg. and t in hours.

Figure 1 is a plot of Eq. 10 and also of Eq. 9. The latter equation with constants evaluated is

$$dW/dt = -4.45\,(1.048 - 0.00245t)^2 \qquad \text{(Eq. 11)}$$

If the implant were cylindrical, then its weight would be given by

$$W = \rho \frac{\pi D^2 h}{4} \qquad \text{(Eq. 12)}$$

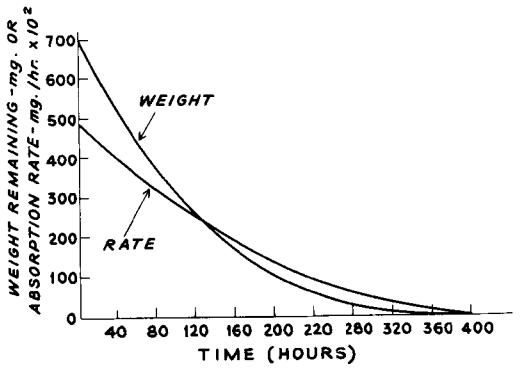


Fig. 1.—Decreases in weight and rate of change of weight as functions of time of an implanted sphere of drug.

where h is its thickness. If the rate of change of thickness and diameter with time were constant then, by same procedure that led to Eq. 3

$$D = D^0 - kt \qquad \text{(Eq. 13)}$$

and

$$h = h^0 - kt \qquad \text{(Eq. 14)}$$

where h^0 is the initial height.

Using Eqs. 13 and 14, Eq. 12 becomes

$$W = \frac{\pi\rho}{4}(D^0 - kt)^2(h^0 - kt) \qquad \text{(Eq. 15)}$$

Determination of the value of k using Eq. 15 is complicated by necessity of obtaining a solution to a cubic equation. However, the solution may be obtained without too much difficulty if a table for the solution of cubic equations, such as that prepared by Salzer, Richards, and Arsham (22), is available.

The rate of change of weight with time is found by taking the derivative of Eq. 15, hence

$$-dW/dt = \rho\frac{\pi k}{4}[(D^0 - kt)^2 + 2(D^0 - kt)(h^0 - kt)] \qquad \text{(Eq. 16)}$$

Equation 15 may be written in terms of the ratio of initial height to initial diameter, *i.e.*, $r = h^0/D^0$, to give

$$W = \frac{\pi\rho}{4}(D^0 - kt)^2(D^0 - kt) \qquad \text{(Eq. 17)}$$

It is of interest to examine the value of Eq. 17 with respect to the value of r. This has been done using data on the absorption of tolbutamide used in the example on absorption from spherical implants. Equation 17 in terms of these data becomes

$$W = 909(D^0 - 0.00245t)^2(D^0 r - 0.00245t) \qquad \text{(Eq. 18)}$$

where W is in mg.

Equation 18 is plotted on Fig. 2 for values of r from 0.1 to 10. This was done by writing Eq. 12 as

$$W^0 = \frac{\rho\pi D^{03} r}{4} \qquad \text{(Eq. 19)}$$

and solving for D^0 at arbitrarily taken values of r. The value of W^0 was that taken from the report previously cited (*loc. cit.*, sphere B). On Fig. 2, all curves shown lie to the left of the curve for a sphere (dotted line) since, for a given material, a sphere has a minimum surface area per unit weight among the various geometric configurations that may be taken. In Fig. 2, the slope of a line at any time is the instantaneous absorption rate. It will be seen in this figure, that for small values of the ratio of thickness to diameter, that absorption rate is nearly constant for most of the time that drug is in implant.

Equations similar to the preceding may be developed easily for other geometric configurations such as oblate and prolate spheroids, etc. Many implants are rod-like with rounded ends, and equations for change in their weight and surface with time would be satisfactorily approximated by the equations for cylinders.

Equation 15 of the preceding has been experimentally verified by Shimkin and co-workers (16) for the case of $h^0 = D^0$. This was done by rearranging this equation to give

$$W^{1/3} = bD^0 - bkt \qquad \text{(Eq. 20)}$$

where $b = (4/\rho\pi)^{1/3}$. Plots of the cube root of weight of implant remaining at various times plotted *vs.* time were linear. They also examined data from work of others wherein $h^0 \neq D^0$ and found that plots of the cube root of weight implant remaining *vs.* time were reasonably linear. Of the various regular geometric objects that would likely be implanted, the implanted cylinder provides the most rigorous test of the assumption made in deriving Eq. 15 and others, *i.e.*, that all dimensions decrease uniformly with time. The work of Shimkin and his co-workers indicates that this assumption is correct (16). The reason that a cylinder provides the most rigorous test is that solution, which is always normal to a surface, is taking place from surfaces normal to each other. The same situation would hold in the case of cubes, for example, but it seems highly unlikely that this object would ever be implanted.

If the intensity of agitation on all faces of a crystalline solid were equal, dimensions would not decrease uniformly if the crystal was anisotropic. This might be a factor to be considered

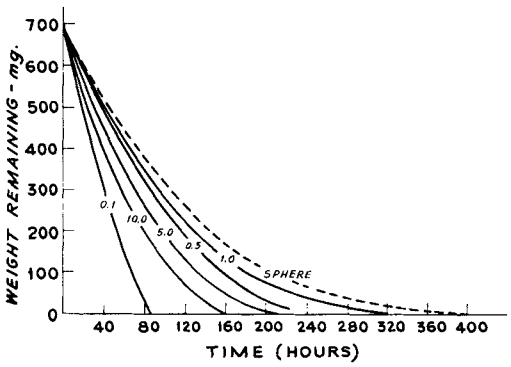


Fig. 2.—Decrease of weight as a function of time for implanted cylinders and a sphere. The numbers by the curves refer to the initial ratio of height to diameter.

with fused implants, but not one with compressed implants. The particles comprising a compressed implant could be expected to be distributed randomly throughout the implant and on its surfaces. The rate of absorption found would be an average representing the arithmetic mean of the rate that would be found for the individual surfaces of the crystal.

It has been reported that absorption of diethylstilbestrol after implantation could be described as a first-order process (23). It seems highly unlikely that this could be the case because absorption of this material is certainly solution rate limited. The result was no doubt an artifact. Many mathematical functions can be roughly fitted to the integrated expression describing a first-order process. For example, if Eq. 15, with constants evaluated for a cylinder with an initial height equal to initial diameter, is used to calculate weight remaining at several times, and values of weight so calculated for the first 100 hours are plotted on semilogarithmic paper as a function of time, the points can be roughly fitted with a straight line. Also, the dimensions given for the apparent first-order rate constant were incorrect.

Body Level of Drug Following Implantation—Mathematical Treatment.—As in the case of any other route of administration, drug absorbed from implant distributes itself in blood and other body fluids and, at the same time, the elimination processes of metabolism and excretion begin. Very little work has been done to express either body level of drug or drug elimination as a function of time in mathematical terms. In many cases, drug elimination may be approximated by considering the process as one whose rate is directly proportional to body level at any time (first order). Assuming this, then the following differential expression should express the rate of change of body level with time when an absorption process is involved

$$\frac{dAb}{dt} = \frac{dA}{dt} - KAb \qquad \text{(Eq. 21)}$$

In Eq. 21, Ab is body level, dA/dt is absorption rate, K is the first-order rate constant for removal of drug from the body, and t is time. If the implant is a sphere, then absorption rate is given by Eq. 9, and if this equation is substituted in Eq. 21, Eq. 22 results

$$\frac{dAb}{dt} = \frac{k\pi\rho}{2}(D^0 - kt)^2 - KAb \qquad \text{(Eq. 22)}$$

Equation 22 may be integrated to give, with constant of integration evaluated at zero time (no drug in the body)

$$Ab = a\left[\frac{(D^0 - kt)^2}{K} + \frac{2k}{K^2}(D^0 - kt) + \frac{2K^2}{K^3} - \left(\frac{D^{0^2}}{K} + \frac{2kD^0}{K^2} + \frac{2k^2}{K^3}\right)\exp\left[-Kt\right]\right] \qquad \text{(Eq. 23)}$$

In Eq. 23 $a = k\pi\rho/2$ and the other terms have been previously defined. The value of the constants in Eq. 23 are all determinable experimentally. Analogous equations based on other implant geometries may also be derived by the procedure shown.

A mathematical treatment similar to the preceding has not been applied to experimentally obtained data. However, drug blood level after implantation of drug has been followed (24–26, 53). In one of these studies (25), pellets of progesterone were implanted both subcutaneously and intrasplenically in mice. Both the free and total progesterone blood levels plateaued after some time following intrasplenic implantation. However, the total progesterone blood level continued to rise after subcutaneous implantation.

In another study, procaine benzylpenicillin tablets were implanted in the vicinity of wounds in human patients undergoing surgery (24). These implantations were either subcutaneous or intramuscular. Penicillin blood level was followed and it was found that, after a few days delay to allow for the attainment of equilibrium, levels fluctuated around a reasonably constant level in a given patient. It was remarked that this method of administering procaine benzylpenicillin was extremely innocuous since no local or general reactions were observed.

In still another study (26), blood level of gold following subcutaneous or intramuscular implantation of pellets of a gold salt in arthritic patients was followed. Measureable quantities of gold were present in blood for many months following implantation.

Absorption and excretion of drug following implantation of spheres has been studied with a different procedure than the one used above to follow absorption (21). It was recognized in this case that absorption from the sphere, which was slow, would eventually become rate limiting in excretion of the drug's metabolite. Thus, for a good part of the time the amount of drug in circulation in the body was small as compared to the amount of drug remaining in solid form in implant and excreted in urine as metabolite. Then the weight of drug in implant at any time should have been given by the Hixon-Crowell cube root law (27) which is

$$W^{0^{1/3}} - W^{1/3} = k't \qquad \text{(Eq. 24)}$$

In Eq. 24, k' was the absorption rate constant whose value depended on solubility of drug at the absorption site, agitation, and other factors. The weight at any time was deducible from the following equation

$$W = W^0 - \frac{Wu}{f} \qquad \text{(Eq. 25)}$$

where Wu was cumulative amount of drug metabolite excreted and f the fraction of a dose of the drug excreted as the metabolite. The quantity Wu was expressible directly in terms of precursor. Substituting Eq. 25 in Eq. 24 gave

$$W^{0^{1/3}} - (W^0 - Wu/f)^{1/3} = k't \qquad \text{(Eq. 26)}$$

When the left hand side of Eq. 26 was plotted as a function of time, a linear relationship was found after a sufficient time had elapsed to allow attainment of equilibrium. The linear relationship indicated the correctness of the assumptions made concerning the absorption, metabolism, and elimination of the drug.

In other work, the kinetics of the excretion of sulfadiazine, following implantation of thin, cylindrical discs of this material in rats, was followed (28). Mean excretion rate over the implantation period was nearly directly proportional to surface area, as estimated by a graphical method (29), at the same time. These results indicated the rate limiting nature of absorption from implants of this drug.

Urinary excretion after implantation of drug has been followed in other work (30). In this instance, 50 mg. of riboflavin with 50 mg. of cholesterol was implanted intramuscularly in two humans. In one patient not deficient in riboflavin, a substantial increase in riboflavin excretion was observed for about 1.5 months. In the other case, the patient was riboflavin deficient and could not be maintained by oral dosage because of severe gastric and intestinal disorders that prevented absorption of this vitamin. The patient could be maintained after parenteral administration of vitamin in solution three times a day, but showed symptoms of deficiency 2 days after cessation of administration. However, after implantation he showed no signs of deficiency for 2 months. Further, his riboflavin excretion was about 10 times greater per day throughout this period than his preimplantation excretion.

Other studies in which drug blood level, excretion of drug or its metabolites, or change in physiological function have been followed after implantation are listed in the Appendix.

Absorption Rate and Surface Area.—It is of interest to consider the relationship between absorption rate and surface area, particularly in regard to the ratio of rate to surface as given in Eq. 27

$$\frac{dA}{dt}\frac{1}{S} = R \qquad \text{(Eq. 27)}$$

In Eq. 27, R is the value of the ratio. For spheres, it is easily shown by Eqs. 4 and 9 that the value of R is given by

$$R = \frac{k\rho}{2} \qquad \text{(Eq. 28)}$$

and R is independent of time.

If the implant is cylindrical in shape, it can be shown by using Eq. 16 and the equation for determining the surface area of this geometric form that the ratio is not a constant, except in one special case, and is, instead, a function of time. This function is given by

$$R' = k\rho\left(\frac{1 + \dfrac{2(h^0 - kt)}{(D^0 - kt)}}{2 + \dfrac{4(h^0 - kt)}{(D^0 - kt)}}\right) \qquad \text{(Eq. 29)}$$

where R' is the function of time. In the special case where initial diameter equals initial height, Eq. 29 reduces to

$$R' = \frac{k\rho}{2} \qquad \text{(Eq. 29a)}$$

and a direct proportionality independent of time exists between absorption rate and surface area of the implant.

A set of widely applied solution rate laws which were written in several forms, depending on initial conditions, are the Hixon-Crowell laws (27). In the several forms of the law, derivations are based on assuming that a direct proportionality exists between surface area and the two-third power of weight of the dissolving substance, which is true among a few common geometric shapes such as spheres, cubes, and cylinders whose initial height equals initial diameter. For the most simple case of dissolution where the object is dissolving in a large enough volume of medium so that the concentration changes may be neglected, the starting equation to derive the law may be written as [a reduced form of the Noyes-Whitney law (31)]

$$dW/dt = -K'S \qquad \text{(Eq. 30)}$$

where K' is constant whose value is a function of several other constants. The physical meanings of these other constants do not bear on the present discussion. When Eq. 30 is rearranged to find the ratio of rate of change of weight (or absorption rate if the process is thought of in terms of rate of change with time of amount of

drug dissolved) to surface area, it will be seen that the resulting equation is of the same form as Eq. 27. If the dissolving solid is a cylinder, then an equation of the same form as Eq. 29 will also express the relationship between rate of weight loss and surface area. Clearly, K' may be a function of time. The Hixon-Crowell laws are in error when any object is considered whose ratio of dimensions change with uniform loss of material from all faces, and this conclusion is explicit from considering Eq. 29.

The reduced form of the Noyes-Whitney law (31) which has been considered in the preceding discussion written for the case of a cylindrical solid undergoing dissolution with uniform loss of all dimensions is, using Eq. 30 and the formula for calculating surface area for a cylinder written in the form that expresses this uniform loss as a function of time

$$-\frac{dW}{dt} = \pi K' \left[\frac{(D^0 - kt)^2}{2} + (D^0 - kt)(h^0 - kt)\right] \quad \text{(Eq. 31)}$$

Equation 31, when integrated, becomes

$$W = \pi K' \left[\frac{(D^0 - kt)^3}{6k} + \frac{(D^0 - kt)(h^0 - kt)^2}{2k} - \frac{(h^0 - kt)^3}{6k}\right] + A \quad \text{(Eq. 32)}$$

where A is the constant of integration. At zero time, $W = W^0$, and when the value of A is evaluated at this time and substituted in Eq. 32 this equation becomes

$$W = W^0 - \pi K' \left[\frac{D^{0^3} - (D^0 - kt)^3}{6k} + \frac{D^0 h^{0^2} - (D^0 - kt)(h^0 - kt)^2}{2k} - \frac{h^{0^3} - (h^0 - kt)^3}{6k}\right] \quad \text{(Eq. 33)}$$

Acidic or Basic Properties and Molecular Weight of the Implanted Drug.—One reason that pellet absorption rate has not been found to correlate well with water solubility, as will be discussed later, is due to the fact the hydrogen ion concentration as well as buffer capacity of the tissue fluids at the site influence the solubility of acidic or basic drugs. These factors have been studied in detail (11).

Absorption rate was assumed to be solution rate limited and expressions derived from Fick's law were obtained to give absorption rate as a function of an acidic or basic drug's dissociation constant, water solubility, and molecular weight. For acidic drugs, experimentally found absorption rates were found to follow, within experimental error, the following equation

$$\frac{dA}{dt} = \frac{cSs}{M^{1/2}}\left(1 + \frac{Ka}{[H^+]_d}\right) \quad \text{(Eq. 34)}$$

In Eq. 34, c is a proportionality constant, M is the molecular weight of a given drug, s is the water solubility of the undissociated acid, Ka the drug's dissociation constant, and $[H^+]_d$ the hydrogen ion concentration of the diffusion layer covering the surface of the implant. This latter quantity was determined by a modification of a method previously used *in vitro* (31). Diffusion layer hydrogen ion concentrations were sometimes substantially different from the hydrogen ion concentrations of the fluids at the implant site, the difference increasing with increasing acidity or basicity of the substances implanted. This is one of the reasons why absorption rate often does not correlate with water solubility.

For basic drugs, the equation appropriate to describe absorption rate was

$$\frac{dA}{dt} = \frac{cSs}{M^{1/2}}\left(1 + \frac{Kb}{[OH^-]_d}\right) \quad \text{(Eq. 35)}$$

In Eq. 35, s is the water solubility of the undissociated base. Equations were also developed for polyfunctional substances (11).

Pellet Density.—Several investigators (14, 32–41) have suggested that pellet density is an important factor influencing absorption rates of implants. It is known that variations in compression pressure used in pellet manufacture affects their densities (or, as some authors put it, their hardness).

Variations in density or compression pressure do affect the weight/area ratio. However, since the absorption rate is directly proportional to the pellet's area exposed to the body fluids at a given time, density alone should not have a direct effect on absorption rate, but only an indirect one in so far as it affects the implant's initial area. In support of this view, Cowie and Folley (17) and Hays, *et al.* (42), have demonstrated *in vivo* that variation of pellet density is a factor of no great importance in implant absorption rate. Parrott, Wurster, and Higuchi (43), correcting for area, have shown *in vitro* that the dissolution rate of spheres was independent of density. They stated that "... although with less dense tablets a greater increment of volume was dissolved per unit time than with the more dense tablets, an equal weight of material was dissolved per unit time from a given surface area for both cases."

In fact, most investigators (17, 44, 45) agree that absorption rates of cast (or fused) and compressed pellets of a given drug are practically the same per unit area. Bishop and Folley (44)

seem to have reversed an earlier claim to the contrary (15).

In more recent work (46) comparing absorption from cast and compressed pellets, it was found that there was no difference when the drug was estradiol. However, compressed desoxycorticosterone acetate pellets were more slowly absorbed than fused pellets. This last result is easily explained if the fusing process produced a more rapidly dissolving polymorphic form of this steroid than the original form that was compressed. Alternatively, the compression process may have produced a more slowly dissolving polymorphic form. Differences in absorption rate between two polymorphic forms of α-methylprednisolone have been observed (11).

In conclusion, the absorption rate and dissolution rate of solid implants are independent of density when corrections are made for area.

Size of Crystals Used in Preparation of Implants.—Soule and Burstein (36) and Greenblatt and Hair (38) asserted that the size of the crystals used in making pellets affects their absorption rate. But Forbes (47) and Hays, *et al.* (42), with experimental evidence, have shown *in vivo* that the size of crystals used in the manufacture of pellets has no effect on absorption rate. The lack of influence of crystal size is most likely due to a factor discussed in a report on some *in vitro* dissolution rate studies (31). Using the equation discussed by Jost (48)

$$Td \cong \sqrt{\eta L/v\rho} \qquad \text{(Eq. 36)}$$

where Td was the thickness of the diffusion layer, η is the viscosity of the medium, L is the linear dimension of surface of solid across which the medium flowed, v was the velocity of flow of the medium as a result of stirring, and ρ was the density of the medium, it was concluded that under the conditions of the experiments, the thickness of the diffusion layer was much greater than the highest point on the pellet's surface (31). Thus, the medium surrounding the pellet was in contact with a diffusion layer of homogeneous composition.

With implanted pellets where the velocity of flow of the medium over the pellet surface is unquestionably much less than the velocity used in the *in vitro* experiments (31), the thickness of the diffusion layer would be large; this can be predicted from Eq. 36. Whether the pellet surface was formed by compression of large or small crystals, or during the fusion process, the diffusion layer is thick enough to mask any of the pellet's minor surface imperfections. Therefore, the absorption rate of compressed pellets should be independent of their method of manufacture, assuming a constant stirring velocity.

Solubility of the Drug.—Deanesly and Parkes (49) were apparently the first to state that the absorption rate of implanted pellets was correlated with the solubility of the substance in body fluids. Others have agreed (32, 33, 50, 51). Biskind, *et al.* (37), stated that the solubility of the compound in various chemical solvents does not influence absorption rate. What they meant by this statement is not clear.

While Lewin and Huidobro (52) could not correlate absorption rate with the water solubility of the drugs they used, they did correlate absorption rate with their experimentally measured serum solubilities.

Influence of Diluents.—It would be expected that the admixture of an inert substance with drug prior to formation of an implant would slow absorption rate of the implant because of the decrease in surface area of drug. On the other hand, it might be expected that, after correction for surface area, diluents could either increase or decrease absorption rate per unit surface area of the implant. If the diluent were a more rapidly dissolving substance than the drug, then drug particles could be expected to be released from the implant with dissolution of the diluent. This process would increase effective surface area of drug. If the drug were a more rapidly dissolving substance than the diluent, then it could be expected that absorption rate per unit area, or even pellet area in some cases, of drug might either remain unchanged or decrease, depending on the relative amounts of drug and diluent. With small amounts of diluent, the diffusion layer would cover the face of the pellet and the absorption site would "see" this area. With large amounts of diluent, as absorption proceeded, drug would have to diffuse to the surface before absorption, and this step could be expected to slow absorption.

The absorption of hexestrol mixed with 25, 49, or 50% lactose has been studied by Cowie and Folley (17). Lactose would be expected to be much faster dissolving than the hexestrol. However, no increase was noted in absorption rate of hexestrol plus lactose as compared to implants of hexestrol alone. With testosterone this was not the case, and the difference in rate became more marked as absorption progressed. This apparent discrepancy can be explained if it is assumed that hexestrol particles were more tightly bound to each other in the implants than were the testosterone particles.

In other work with lactose as a diluent, in amount of 39 to 49%, for hexestrol and diethyl-

stilbestrol, it was stated that absorption was "probably less rapid" with diluent present (54).

Absorption studies of various steroids from implants containing cholesterol have given some unexpected and surprising results. Some of this work has been briefly reviewed by Fuenzalida (55). When a number of steroids were mixed with cholesterol, the absorption of the steroids per unit surface area of drug was very markedly decreased. Further, cholesterol by itself, which is absorbed so slowly that its rate is difficult to measure even in implant studies, was absorbed at measurable rates when a number of steroids were admixed with it. During the course of absorption, the cholesterol-drug ratio did not change (55). These results strongly suggest compound formation between the steroids and cholesterol. It is known that cholesterol forms 1:1 compounds with β-sitosterol, vitamin D_2, and other structurally similar substances, as well as a variety of other substances (56).

Paraffin wax has been used as a diluent to slow absorption of implanted drug (57, 58). Most of the other studies with implants containing inert diluents are listed in the Appendix under cholesterol or the sugars, lactose and glucose. From results obtained in work with implant mixtures, as discussed above and listed in the Appendix, it is rather clear that little quantitative information on the role of the diluent exists.

Phagocytosis.—Forbes (14) and Foss (32) supposed that pellet absorption might be connected with the process of phagocytosis. But after Lewin and Huidobro (52) reviewed the literature on this point, they demonstrated experimentally that implants could be absorbed without the occurrence of phagocytosis. This factor, therefore, does not appear to be important.

Physiological Need of the Animal.—Neither physiological need for the implanted substance nor the animal's sex seems to affect the absorption rates of implants (14, 33, 34, 38, 40, 42, 47, 59–66). These experiments and observations lend support to the view that absorption rate is solution rate limited in the usual case. In the nonsolution rate limited case, absorption rate varies with the difference between the concentration of drug in the implant's diffusion layer and the concentration in the fluids at the membrane in contact with it. The physiological need of an animal might control the concentration of drug in the fluids at the membrane in some cases, but it is explicit in solution rate limited absorption that this concentration is negligible in comparison to the concentration of drug at the pellet's surface. Cholesterol implants serve as a good example of a nonsolution rate limited case of absorption (11).

Site of Implantation and Body Movement.—Statements in the literature regarding absorption rates of implants at various sites in the body are not in agreement. Some authors (14, 36, 38, 50, 51, 54, 67) say that absorption site is important, while others (40, 68, 69) say that absorption rate is essentially independent of the site of implantation. Unfortunately, the statistically significant key experiment which would resolve these opposing views has not been conducted. Reported absorption rates, in the main, are based on results from too few pellets and animals, and the many possible implantation sites have not been systematically studied. It would seem that site of implantation should influence absorption rate because degree of agitation may be different at different sites.

Variations in absorption rate are observed even when a single, carefully defined anatomical region is implanted. Absorption rate varies inversely with the thickness of the diffusion layer surrounding the implant's surface. The thickness of this fluid layer in a particular animal can change with a variation in stirring rate, either due to animal movement or change in flow of interstitial fluid in the region of the pellet.

The clinical variations in absorption rates of desoxycorticosterone acetate pellets observed by McCullagh, *et al.* (70), might well be explained by stirring, and its effect on the thickness of this liquid layer. In this connection, Kearns (71) suggests that the patient massage the area over the implant to increase the rate of absorption.

Encapsulation.—After some drug pellets are implanted for a time, they become surrounded by a fibrous capsule whose morphology and histology have been described (14, 41, 72–75, 84). While many claim that such capsules retard the implant absorption rate (32, 36, 39, 51, 61, 72, 76–83), others think it has no effect (15, 16, 42, 50, 85).

Although no completely convincing experiment has settled this question, the latter view is probably correct because of the solution rate limited nature of absorption from implant. There is evidence (32) that the more rapidly absorbed substances have thicker capsules than the more slowly absorbed ones. Very rapidly absorbed substances do not form capsules, because there is not time enough for the body to form them. The view, which follows, by Shimkin *et al.* (16), seems correct: ". . . it is just as likely that the thickness of the capsule is determined by the slow absorbability of the material rather

than the slowness of absorption being due to the deposition of the surrounding fibrous tissue."

Until more quantitative work is available relating absorption rate of pellets to capsule formation, encapsulation can be disregarded as a factor of major importance.

"Ghost" Formation.—The term "ghost" was coined by Folley (82) who observed that proteinaceous material had infiltrated into the pores of compressed implants. "Ghost" formation was subsequently studied in more detail (15, 17, 42, 45, 52, 54, 65, 83, 86, 87). Some believe that the presence of a "ghost" affects absorption rate (15, 17, 42, 54, 65, 87), while others claim it does not (45, 52). Folley (65), who used hexestrol pellets of differing weights, noted that for small pellets (50 mg.) the correction for the "ghost's" weight made no detectable difference in the absorption rate.

For precise measurements of absorption rate there is no doubt that the weight of the "ghost" should be considered. But whether the "ghost" actually inhibits absorption rate due to plugging of the pellet pores, as some claim, is open to question. On this point two lines of evidence suggest that the "ghost" does not affect absorption rate. (*a*) The absorption rates of cast and of compressed pellets *in vivo* are generally agreed to be the same, as previously discussed, in spite of the fact that the compressed pellet histologically has the more extensive "ghost." (*b*) The *in vitro* studies of Wurster and Seitz (88) have shown that the pores in pellets compressed in air are largely occluded by air and are almost entirely unavailable to the aqueous solvent. One might suppose, then, that the absorption rate of a pellet whose pores are occluded by air or by proteinaceous material would be almost identical.

"Ghost" formation as a factor influencing absorption rate can be considered unimportant. The weight of the "ghost," however, should be accounted for when the weights of pellets are measured after implantation.

Animal's Age.—Forbes (62) observed that the absorption rate of testosterone propionate pellets implanted into young rats decreased during the first 2 months of life. This effect of age on absorption rate may be due in part to the fact that the newborn rat is more responsive to changes in external temperature than older rats (89), and the solubility and rates of diffusion of substances are directly related to temperature. In fact, Deanesly (63) hinted at this when she attributed the different absorption rates of estrone pellets in rats and mice to differences in body temperature or metabolism. Animal physical activity is also a function of the animal's age.

Species Difference.—Very little work has been done to determine species difference in absorption rate. The observation has been made that rats absorb certain drugs after implantation more rapidly than man (46). A comparative study has been done using albino rats, desert rats, and golden hamsters (90). Rate of absorption decreased in the order listed. Species differences are no doubt due to differences in physical activity, body temperature, and composition of the body fluids at the implantation sites.

SUMMARY

Factors that clearly affect absorption rates of implants are their surface area and solubility in body fluids. Factors proposed that do not affect absorption rate greatly (if at all), with the present information, are pellet density (when area corrections are made), crystal size used in implant preparation, phagocytosis, physiological need or sex of the animal, encapsulation, "ghost" formation, and the age of the animal (if it is not young). Factors such as site of implantation and body movement and diluents do have an effect, but little quantitative information is available concerning the magnitude of the effect.

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Research Articles

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By SALAH ELDIN F. ALI† and EARL P. GUTH

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IT IS WELL known that on harvesting an entire plant or removing parts from the plant, many of the vital processes do not stop immediately (1). It is also known that the length of time and the degree of temperature required for drying plant materials affect the rate and intensity of these processes and, consequently, could affect the nature of some constituents normally present. The chemical reactions which occur in plant cells are apparently accelerated by enzymes. Therefore, as long as conditions are favorable to enzymatic action, these reactions will proceed. The rate at which an enzymatic reaction proceeds is influenced not only by the temperature, but also by the length of time that the reaction mixture has been maintained at that temperature. Within limits, an increase in the

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Accepted for publication February 9, 1962.
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Research Articles

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IT IS WELL known that on harvesting an entire plant or removing parts from the plant, many of the vital processes do not stop immediately (1). It is also known that the length of time and the degree of temperature required for drying plant materials affect the rate and intensity of these processes and, consequently, could affect the nature of some constituents normally present. The chemical reactions which occur in plant cells are apparently accelerated by enzymes. Therefore, as long as conditions are favorable to enzymatic action, these reactions will proceed. The rate at which an enzymatic reaction proceeds is influenced not only by the temperature, but also by the length of time that the reaction mixture has been maintained at that temperature. Within limits, an increase in the

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temperature increases the rate of enzymatic reactions. When the optimum temperature is exceeded, there is a decrease in the rate of these reactions because of a denaturation effect upon the enzyme (2–4).

Since high temperatures do affect some plant materials during the process of their drying, it would be reasonable to dry these materials at low temperatures. The freeze-drying process has proved to be feasible for drying and preserving plant and animal products and the application of this method as a means of drying crude drugs and drug extracts has been employed by many investigators (5–10).

The purpose of this study is to investigate the possibility that certain plant principles which are now known may not necessarily be the actual constituents of that source. In order to achieve this objective, an attempt was made to examine specifically some of the chemical changes which may occur after the plant has been harvested and dried.

This paper is concerned with the differences found in the lipid fraction of dandelion root (*Taraxacum officinale*).

EXPERIMENTAL

Collection and Drying.—The roots of dandelions were gathered at three different seasons; Autumn, Spring, and Summer, from a field adjacent to the Ohio State University.

The roots were gathered and divided in two lots each time; one lot was immediately frozen with dry ice.

The unfrozen roots were cleaned and then dried in an oven at 50°; these were designated as O.D. samples. The frozen roots were rapidly cleaned and freeze-dried at a temperature not higher than 25° and a pressure of 100 to 200 μ of mercury; these were designated as F.D. samples.

Determination of Fatty Acids.—The lipid-containing fraction of the roots was extracted with ether in a Soxhlet apparatus. The ether was then removed by vacuum distillation, the extracts dried in a desiccator, and stored under nitrogen at 0° in a tightly closed container.

Preparation of the Methyl Esters of the Fatty Acids.—The dried ether extract was saponified with 10% alcoholic potassium hydroxide. After the nonsaponifiable matter was removed, the soap solution was acidified with 25% sulfuric acid and washed with ether. The ether was removed by vacuum distillation.

The fatty acids so obtained were dissolved in anhydrous ether and methyl esters prepared using diazomethane.

The constituent fatty acids were tested and identified, as their methyl esters, using gas-liquid chromatography.[1] The gas-chromatographic apparatus employed in this study was a Wilkens Areograph model A-110-C.

[1] These findings were confirmed by Mr. Robert B. Iden, Battelle Memorial Institute, Columbus, Ohio.

Preferred Operating Conditions.—To minimize the effects of the many operative variables, the following conditions were established: a column packed with 10% diethylene glycol succinate on 60–80 mesh firebrick; column dimension, 72 in. × 0.25 in. (i.d.); flow rate, 90 ml./min. helium; temperature, 185° for the resolution of low molecular weight fatty acid methyl esters and 195 to 198° for the resolution of higher molecular weight fatty acid methyl esters. The linearity of the plot of the logarithm of apparent retention volume (V_R') *vs.* the chain length for a number of known saturated straight-chain fatty acid methyl esters was used as an indication of constant column temperature for each analysis.

Identification of Fatty Acids.—The retention volume (V_R') of each component fatty acid was precisely measured to the nearest 0.1 cm.

The preliminary characterization of the fatty acid components of the lipid fraction in each of the oven-dried and freeze-dried root, collected at different seasons, was obtained by simple comparison of their apparent retention volume with those of known saturated fatty acid methyl esters from a standard curve prepared under similar conditions.

The identification of the peaks was further confirmed by the addition of a known synthetic mixture of pure fatty acid methyl esters that correspond to the suspected peaks to the solution of the unknowns, and a new chromatogram was obtained. The results and the relative proportions of the fatty acid methyl esters separated from the lipid fraction of the root are listed in Table I.

TABLE I.—COMPONENT FATTY ACIDS OF LIPID FRACTION OF FREEZE-DRIED AND OVEN-DRIED DANDELION ROOT, SPRING COLLECTION

Fatty Acid	Chain Length	Relative Proportion, % O.D.	F.D.
Caprylic	C_8	trace	...
Capric	C_{10}	trace	...
Lauric	C_{12}	0.29	0.3
Myristic	C_{14}	3.94	5.1
Unknown 1	...	0.56	0.5
Unknown 2	...	0.23	0.4
Palmitic	C_{16}	21.29	20.2
Unknown 3	...	0.30	0.8
Unknown 4	...	0.28	0.3
Stearic	C_{18}^{5}	1.03	1.8
Oleic	$C_{18}^{1=}$	3.95	4.1
Unknown 5	...	0.00	0.2
Linoleic	$C_{18}^{2=}$	44.89	40.4
Unknown 6	...	1.14	1.0
Linolenic	$C_{18}^{3=}$	21.42	16.7
Unknown 7	...	0.67	4.1
Unknown 8	...	trace	4.1

DISCUSSION

The neutralization number of the crude ether extracts of the root (Table II) indicate that there was a significant increase in the saponification number of the ether extract of freeze-dried Summer root as compared with the corresponding oven-dried sample. This would indicate that the concentration of low molecular weight fatty acids in the extracts obtained from the freeze-dried sample was higher than those present in the extracts obtained

TABLE II.—SAPONIFICATION NUMBER OF THE LIPID FRACTION OF THE FREEZE-DRIED AND OVEN-DRIED DANDELION ROOT (U.S.P. XVI)

Sample	Autumn O.D.	Autumn F.D.	Spring O.D.	Spring F.D.	Summer O.D.	Summer F.D.
1	111.5	90.8	124.5	123.6	152.1	276.3
2	105.2	96.3	123.7	118.5	159.3	280.5
3	110.2	99.4	119.3	123.3	166.1	281.6
Av.	108.9	95.5	122.5	121.8	159.2	279.4

from the oven-dried sample. Extracts from the oven-dried Autumn and Spring collections showed a slight increase in saponification numbers over those obtained from the freeze-dried samples. Such differences between crude products are not usually considered to be reliable. Therefore, no immediate interpretation was made, the composition and degree of purity of the extracts being unpredictable. A negative iodine number was obtained for the ether extract prepared from the freeze-dried Summer root as compared with the extract prepared from the oven-dried sample. The iodine number of the extracts prepared from the oven-dried Autumn and Spring roots indicated the existence of a relatively higher degree of unsaturation as compared with the corresponding freeze-dried samples.

The distribution of fatty acids (Table III and Fig. 1) found in the freeze-dried and oven-dried root of the Spring collection shows some interesting differences. Myristic and stearic acids appear to be slightly greater in the freeze-dried sample. In addition, unknown fatty acids Nos. 2, 3, and 7 are greater in the freeze-dried sample. Unknown fatty acids Nos. 5 and 8 appear only in the freeze-dried sample. On the other hand, palmitic, linoleic, and linolenic acids appear in greatest quantity in the oven-dried sample.

Myristic, palmitic, and oleic acids appeared to be greatly increased (Fig. 2 and Table IV) in the freeze-

TABLE III.—IODINE NUMBER OF THE LIPID FRACTION OF THE FREEZE-DRIED AND OVEN-DRIED DANDELION ROOT (U.S.P. XVI)

Sample	Autumn O.D.	Autumn F.D.	Spring O.D.	Spring F.D.	Summer O.D.	Summer F.D.
1	64.5	65.9	99.7	89.5	52.2	...
2	63.1	57.0	98.2	93.5	54.1	...
Av.	63.8	61.5	98.9	91.5	53.2	...

TABLE IV.—RELATIVE DISTRIBUTION OF SOME IMPORTANT FATTY ACIDS OF FREEZE-DRIED AND OVEN-DRIED DANDELION ROOT, AUTUMN COLLECTION

Fatty Acid	Chain Length	Relative Proportion, % O.D.	Relative Proportion, % F.D.
Myristic	C_{14}	5.24	15.06
Palmitic	C_{16}	31.66	37.63
Oleic	$C_{18}{}^{1=}$	3.14	4.68
Linoleic	$C_{18}{}^{2=}$	47.43	33.86
Linolenic	$C_{18}{}^{3=}$	12.77	8.70

dried sample of the Autumn collection. Linoleic and linolenic acids are found to be considerably greater in the oven-dried sample of this collection.

Unknown fatty acid No. 1 (Fig. 3) appeared in greatest quantity in the freeze-dried root of the Summer collection, while it appeared only in a minute quantity in the oven-dried sample. This increase may explain, in part, the reason for a higher saponification number of the freeze-dried Summer sample over that of the corresponding oven-dried samples.

The presence of traces of caprylic and capric acids in the oven-dried sample of the Spring collection is also indicated by a slight increase in the saponification number of the ether extract obtained from the roots that were oven-dried over those of the corresponding freeze-dried sample. The appearance of these two acids in the oven-dried samples, while none appear in the freeze-dried sample, may be due to loss of these fatty acids during the freeze-drying process, since this was performed at a pressure of 200 μ. On the other hand, this difference might represent a breakdown of larger fatty acids due to higher temperature in the oven-drying process.

In view of these findings, it might be reasonable to assume that the decrease in the quantities of myristic acid in the lipid fraction of the oven-dried samples of the Spring and Autumn roots as compared with the freeze-dried samples indicates a continued synthesis of fatty acids in the oven-dried samples. Such process may be blocked by freeze-drying the root. This is also indicated by a considerable decrease in palmitic acid content of the oven-dried Autumn samples as compared with the corresponding freeze-dried samples. The loss in

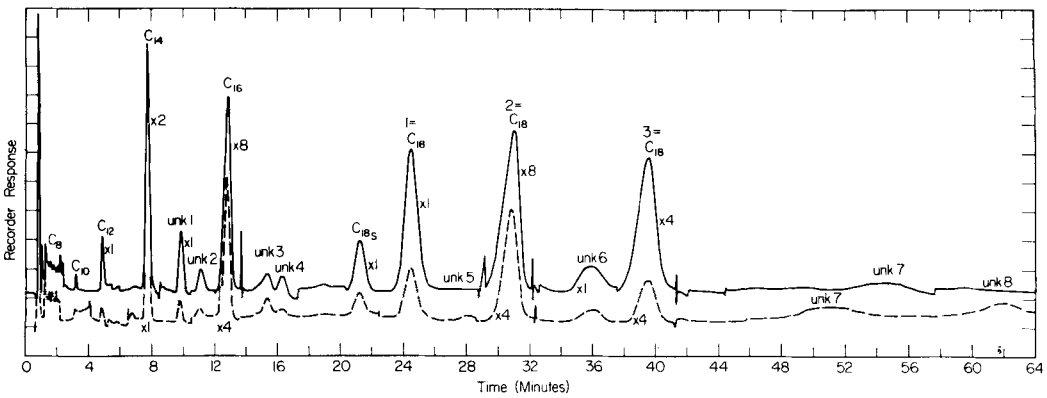


Fig. 1.—Separation of methyl esters of fatty acids in freeze-dried and oven-dried dandelion root at 196°, Spring collection. Samples: —— (B) 4.0 μl. O.D., – – (A) 5.7 μl. F.D.; column packing, 10% E.G.S. on firebrick; column, 144 in. × 0.25 in.; flow rate, 105 ml./min.; temp., 196°; current, 236 mv.

stearic acid of the oven-dried samples of the Spring collection and that of oleic acid in the oven-dried samples of the Spring and Autumn collection tends to support this conclusion.

In the oven-drying process, the Spring collected root shows a higher content of palmitic, linoleic, and linolenic acids over the freeze-dried sample, which seems to indicate that synthesis of the fatty acids continues in the oven-drying process. It is further believed that little degradation occurs in the oven-drying process since, if this did occur, there should be less of these larger fatty acids and a greater percentage of smaller fatty acids. The data (Table I) show, however, that there is a greater percentage of small molecular weight fatty acids and a smaller percentage of the larger fatty acids in the freeze-dried sample. This finding also supports the contention that synthesis does continue for a time during the oven-drying process. This is further supported by the fact that these differences are more marked

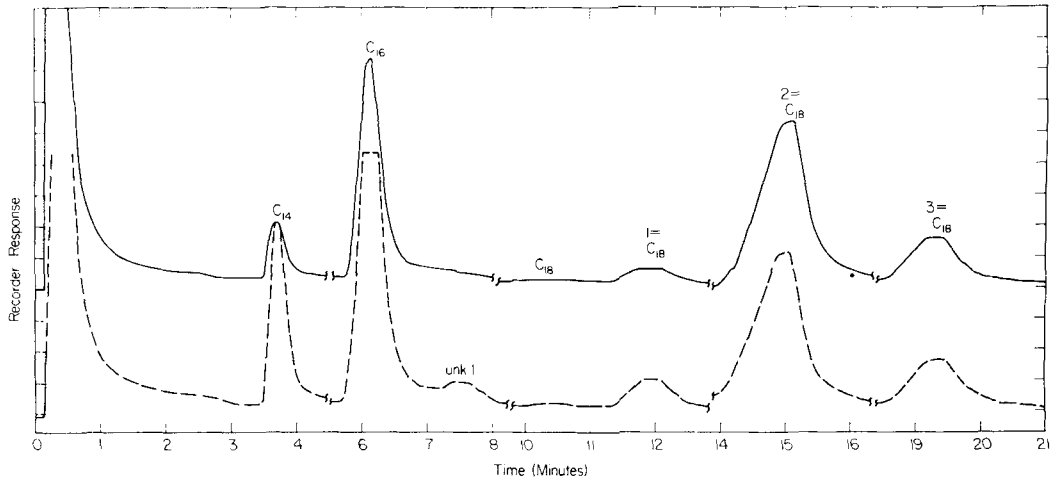


Fig. 2.—Separation of methyl esters of fatty acids in freeze-dried and oven-dried dandelion root at 195°, Autumn collection. Samples: ——(B) 5.0 μl. O.D., – –(A) 5.0 μl. F.D.; column packing, 10% E.G.S. on firebrick; column, 72 in. × 0.25 in.; flow rate, 90 ml./min.; temp., 195°; current, 220 mv.

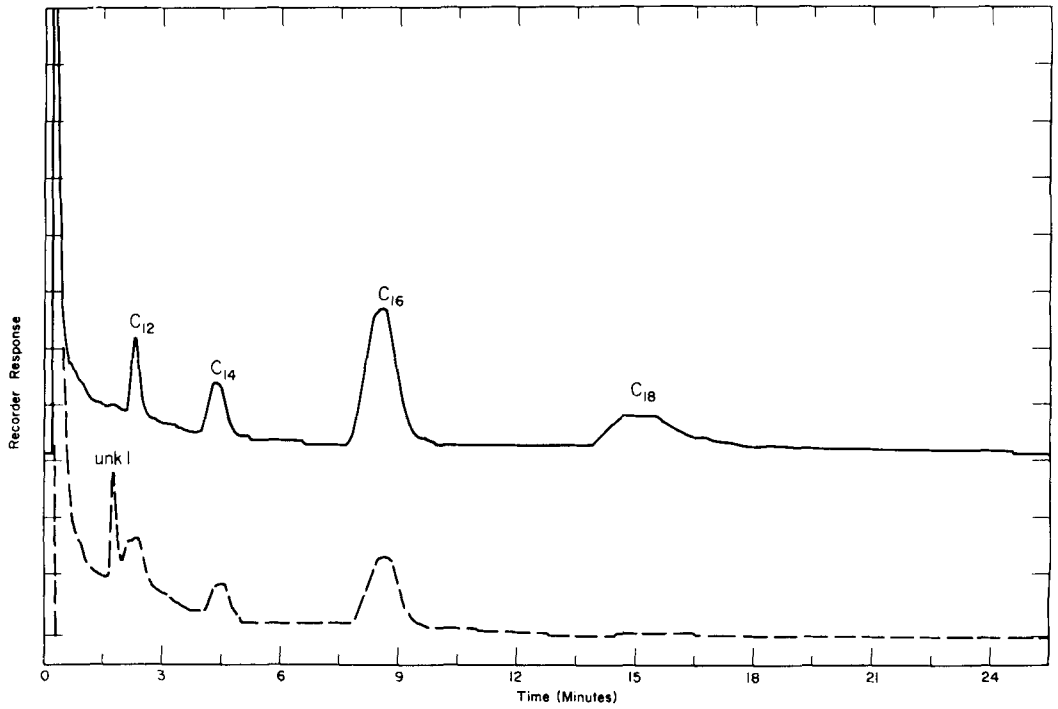


Fig. 3.—Separation of methyl esters of fatty acids in freeze-dried and oven-dried dandelion root at 198°, Summer collection. Samples: ——(B) 8.0 μl. O.D., – –(A) 6.5 μl. F.D.; column packing, silicon on firebrick; column, 72 in. × 0.25 in.; flow rate, 90 ml./min.; temp., 198°; current, 220 mv.

in the Autumn collection which required 6 days to oven-dry as compared with the Spring collection which was dried in 3 days.

Unknown acids Nos. 1, 2, 3, 4, and 6 may possibly be branched-chain, unsaturated, or odd-numbered fatty acids of different chain length. The difference between the quantities of these fatty acids in the oven-dried and freeze-dried samples can also be explained in reference to the amounts of the larger fatty acids present.

The appearance of unknown acids Nos. 5 and 8 only in the freeze-dried samples of the Spring root is of special interest. It was observed that the solutions of methyl esters of fatty acids of the freeze-dried samples were unstable. After a short time, the solutions developed a white precipitate. Consequently, fresh solutions were always used in the gas chromatographic determination of the freeze-dried samples. The solutions of the esters of the oven-dried samples were apparently stable in that they did not show any precipitation over a 12-hr. period. This observation suggests the possibility that unknown acids Nos. 5, 7, and 8 may be highly unsaturated compounds or otherwise unstable molecules which could have been destroyed in the oven-dried samples.

The alteration in the fatty acid distribution, as illustrated by this work, could suggest that other components of this plant are altered by methods used in curing the plant.

SUMMARY AND CONCLUSIONS

1. The fatty acid contents are greatly affected by the temperature and time required for drying dandelion root.

2. Oven-drying does greatly influence the distribution and molecular nature of fatty acid components of dandelion root as compared to freeze-drying.

3. It appears that myristic and stearic acid contents are higher in the freeze-dried than in the oven-dried dandelion root of the Spring collection. Myristic, palmitic, and oleic acids are higher in the freeze-dried than in the oven-dried root of the Autumn collection.

4. In oven-drying process, the Spring and Autumn collected roots show a higher content of linoleic and linolenic acids over the freeze-dried samples, which seems to indicate that synthesis of fatty acids continues in the oven-drying process.

5. It is believed that little degradation occurs in the oven-drying process since, if this did occur, there should be less of these larger fatty acids and a greater percentage of smaller fatty acids.

6. The presence of a greater percentage of small molecular weight fatty acids and a smaller percentage of the larger fatty acids in the freeze-dried samples suggests that synthesis does continue for a time during the oven-drying process.

7. Unknown fatty acids Nos. 2, 3, and 7 are greater in the freeze-dried root of the Spring collection than in the oven-dried samples. Unknown fatty acids Nos. 5, 7, and 8 appear only in the freeze-dried root.

8. Unknown fatty acids Nos. 1, 2, 3, 4, and 6 may possibly be branched-chain, unsaturated, or odd-numbered fatty acids of different chain lengths.

9. Unknown acids Nos. 5, 7, and 8 may possibly be highly unsaturated compounds.

10. The changes occurring in the fatty acid distribution of the root as a result of the two methods of drying indicate that the method of drying a plant material can have a marked effect on these molecules.

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Isotonic Solutions XIII

Hemolysis of Red Corpuscles by Various Substances in the Presence of Sodium Chloride

By MARGARET A. SHAW† and WILLIAM J. HUSA

Various compounds which cause hemolysis in the presence of 0.6% sodium chloride were studied in environments of higher concentrations of sodium chloride. An increase in the concentration of sodium chloride reduced the degree of hemolysis produced by a compound in the presence of 0.6% sodium chloride in some instances, but this effect did not occur as a general rule for all compounds. Since 0.1% sodium carbonate did not cause an appreciable discoloration of oxyhemoglobin, it was retained as a standard for total hemolysis in the hemolytic method employed.

PREVIOUS INVESTIGATORS have emphasized the uncertainty associated with the use of physicochemical data to determine whether a solution is isotonic with the blood since methods utilizing colligative properties of solutions ignore the selective permeability of the corpuscular membrane and other pertinent factors. This variation is vividly shown by the fact that some substances cause hemolysis even in the presence of 0.6% sodium chloride which would prevent hemolysis if no other substance were present (1–3).

Compounds studied in this investigation generally exhibited a common property in their behavior toward red blood corpuscles when examined in the presence of 0.6% sodium chloride, *i.e.*, at a certain point, a relatively small change in concentration of the drug generally caused an abrupt increase in the degree of hemolysis. Exceptions to this were procaine hydrochloride and 2-propoxyprocaine hydrochloride.

Marcus and Husa (2) found that solutions of procaine hydrochloride ranging in concentration from 4.00 to 10.00% caused hemolysis in the presence of 0.6% sodium chloride. Using the hematocrit method, Setnikar and Temelcou (4) found that a solution of 5.05% procaine hydrochloride in 0.6% sodium chloride produced hemolysis of rabbit erythrocytes, but hemolysis was prevented when the sodium chloride concentration was increased to 1.3%.

The purpose of the present investigation was to determine by use of the hemolytic method whether the presence of hyperosmotic concentrations of sodium chloride might have general application in preventing the destruction of red blood cells by substances which cause hemolysis in an environment of 0.6% sodium chloride. In addition, a recent report (5) indicated that the alkalinity of 0.1% sodium carbonate solution caused a discoloration of oxyhemoglobin which would invalidate its use as a standard for 100% hemolysis in the hemolytic method. Five hemolyzing solutions were compared in regard to the degree of hemolysis each produced and to determine whether there was any significant change in the color of oxyhemoglobin upon mixing with each hemolyzing solution.

EXPERIMENTAL

Collection of Blood.—The human blood used in various experiments was obtained primarily from the veins of the arms of a 27-year old white female by means of a hypodermic syringe. Blood was taken occasionally from other white volunteers, and a fresh blood sample was utilized for each experiment. Each fresh blood sample was treated in the manner previously described by Grosicki and Husa (6).

Chemicals.—The medicinal chemicals were contributed by the manufacturers. The other chemicals were of C.P. grade with the exception of saponin which was a purified grade.

Preparation of Solutions.—Solutions were prepared by diluting aliquot portions of appropriate stock solutions with triple distilled water.

Quantitative Determination of Per Cent Hemolysis.—Determination of the degree of hemolysis produced by various solutions under examination was accomplished by use of the hemolytic method described by Grosicki and Husa (6). Fragility tests are used to minimize deviations due to the variability of blood when hemolysis is due entirely to osmotic effects. However, no standard method has been established to compensate for the variability of blood when some factor such as an effect on the corpuscular membrane is operative in the presence of 0.6% sodium chloride which would prevent hemolysis if no other compound were present. Consequently, fragility tests offered no advantage in

Received May 29, 1961, from the College of Pharmacy, University of Florida, Gainesville.
Accepted for publication January 11, 1962.
This paper is based in part upon a dissertation presented by Margaret A. Shaw to the Graduate Council of the University of Florida in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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the majority of experiments performed in this investigation and were deemed unnecessary in obtaining information which would reveal whether or not the addition of hyperosmotic sodium chloride concentrations would prevent hemolysis by certain compounds which cause hemolysis in the presence of 0.6% sodium chloride. With the elimination of the fragility tests, solutions of sodium chloride alone, corresponding to the sodium chloride concentrations utilized in a particular experiment, were used as controls.

Comparison of Hemolyzing Solutions Producing Approximately Complete Hemolysis.—The hemolyzing solutions tested exhibited no appreciable degree of absorbance *per se*. The hemolytic method was employed to compare the degree of hemolysis elicited by triple distilled water, 0.1% sodium chloride, 0.1% sodium carbonate, 0.01% saponin, and a solution containing 0.1% sodium carbonate and 0.01% saponin. The greatest difference in the average colorimeter readings was 1.3% when per cent hemolysis was calculated on the basis of triple distilled water producing complete hemolysis; this difference was considered to be within experimental error. In other tests, colorimeter readings were determined immediately after mixing each of the hemolyzing solutions with oxyhemoglobin solution prepared by laking red blood cells in triple distilled water. Colorimeter readings were also taken after storage in a refrigerator for 4- and 24-hour periods. Results indicated that no appreciable discoloration of oxyhemoglobin occurred within the time interval required for hemolytic determinations. Consequently, 0.1% sodium carbonate solution was retained as a standard for complete hemolysis in the hemolytic method employed in this investigation.

Reproducibility of Results.—Parallel results and duplicate colorimeter readings were generally obtained when the degree of hemolysis approximated 0 or 100%. However, experimental data associated with concentrations of some compounds producing an intermediate degree of hemolysis exhibited excessive variations, which might be attributed to factors the hemolytic method employed, is incapable of controlling. However, ascertaining the exact percentage of hemolysis produced by the compounds was not essential in determining whether or not the addition of hyperosmotic quantities of sodium chloride would prevent hemolysis produced by the compounds in the presence of 0.6% sodium chloride.

Experiments were conducted to determine whether an uncontrollable factor might be introduced in hemolytic determinations during the time lapse between the addition of blood to the solution under study and the subsequent inversion of the colorimeter tubes to insure thorough mixing. Experiments were performed in which the solutions under study, as well as the 0.85% sodium chloride blank and 0.1% sodium carbonate standards for 100% hemolysis, were treated in the same manner with the exception that one portion of the tubes was shaken immediately after the addition of blood, whereas their counterparts were treated in the usual way. Results indicated that the time lapse between the addition of blood and shaking of the mixture did not account for the variations found in duplicate tubes.

TABLE I.—DEGREE OF HEMOLYSIS PRODUCED BY VARIOUS CONCENTRATIONS OF COMPOUNDS IN VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE[a]

Compound	Per Cent Compound	Degree of Hemolysis[b] NaCl, % 0.6	0.9	1.2	1.5
Procaine HCl	4.00	L	–	–[c]	–
	6.00	M	M	–[c]	–
	10.00	M	M	–[c]	–
2-Propoxy-procaine HCl	1.00	–	–	–	–
	2.00	M	–	–	–
	7.00	–	–	–	–
NH_4 Salicylate	1.00	–	–	–	–
	2.20	M	–	–	–
	2.60	H	M	M	L
NH_4 Benzoate[d]	2.40	–	–	–	–
	3.20	M	L	L	L
	4.00	H	H	H	H
Pramoxine HCl[d]	0.10	–	–	–	–
	0.12	M	L,M	L,M	M
	0.15	H	H	H	H
Benoxinate HCl[d]	0.10	–	–	–	–
	0.30	L,M	L	L	–
	0.90	H	H	H	H
Hexylcaine HCl[d]	0.70	L,M	–,L	–,L	–
	0.90	M	M	L,M	M
	2.00	H	H	H	H
Butacaine SO_4[d]	0.30	–,L	–,L	–,L	–,L
	0.35	L,M	L,M	L,M	L,M
	0.50	H	H	. . .[e]	. . .[e]
Phenmetrazine HCl[d]	3.00	L,M	L,M	L,M	M
	4.20	M	M	M,H	H
	5.40	H	H	H	H

[a] Results of two blood samples unless otherwise indicated. [b] –, no hemolysis; L, <15%; M, 15–85%; H, >85%. [c] Determined in 1.3% sodium chloride. [d] Results of four blood samples. [e] Precipitation before addition of blood.

DISCUSSION

Higher concentrations of sodium chloride generally caused a significant reduction in the degree of hemolysis produced by procaine and 2-propoxyprocaine hydrochlorides, ammonium salicylate, and ammonium benzoate in the presence of 0.6% sodium chloride. In most cases, hemolysis was eliminated. However, a smaller inhibitory action was exhibited by solutions of ammonium benzoate. Furthermore, a notable exception was apparent in 4.00% solutions of ammonium benzoate which caused approximately total hemolysis in environments of 0.6, 0.9, 1.2, and 1.5% sodium chloride as indicated in Table I. Absence of hemolysis in procaine hydrochloride solutions ranging in concentration from 4.00 to 10.00% in the presence of 1.3% sodium chloride indicates that the results obtained by Setnikar and Temelcou (4) for rabbit blood also hold true for human blood.

Two trends appeared evident upon examination of the experimental data obtained for pramoxine, benoxinate, and hexylcaine hydrochlorides, and butacaine sulfate. Results generally indicated that additional quantities of sodium chloride inhibited hemolysis somewhat when concentrations of the drug were used such that the degree of hemolysis produced in the presence of 0.6% sodium chloride was low. However, in concentrations of the compound causing degrees of hemolysis ranging from intermediate to essentially complete in 0.6% sodium chloride, the addition of hyperosmotic quantities of sodium chloride did not appear to inhibit hemolysis.

A crystalline precipitate appeared in solutions of several concentrations of butacaine sulfate in 1.2 and 1.5% sodium chloride before the addition of blood. In contrast to the effect produced on butacaine sulfate solutions by increased proportions of sodium chloride, several solutions of pramoxine hydrochloride in some concentrations of sodium chloride exhibited evidence of precipitation after incubation and centrifugation of the blood-salt mixtures. Higher concentrations of sodium chloride appeared to decrease the precipitation until the effect was eliminated as far as visual observation could detect.

Experimental data indicated that phenmetrazine hydrochloride did not behave in a manner similar to any of the other compounds studied in this investigation. As the concentration of sodium chloride was increased, a corresponding augmentation in the degree of hemolysis, compared to that produced in the presence of 0.6% sodium chloride, was effected. The only exception to this trend was exhibited by 5.40% phenmetrazine hydrochloride solutions in environments of 0.6, 0.9, 1.2, and 1.5% sodium chloride; essentially complete hemolysis was elicited in the presence of 0.6% sodium chloride, and increasing the concentration of sodium chloride afforded no apparent change in the degree of hemolysis.

SUMMARY

1. An increase in the concentration of sodium chloride reduced the degree of hemolysis produced by procaine hydrochloride, 2-propoxyprocaine hydrochloride, and ammonium salicylate solutions in an environment of 0.6% sodium chloride. A similar effect was noted in most of the concentrations of ammonium benzoate studied.

2. Increasing the concentration of sodium chloride in solutions of pramoxine, benoxinate, and hexylcaine hydrochlorides, and butacaine sulfate afforded some reduction in the degree of hemolysis produced by the compound in 0.6% sodium chloride in cases where the degree of hemolysis was slight. No reduction in the degree of hemolysis was observed as a result of augmenting the sodium chloride concentration when the compound under examination caused degrees of hemolysis ranging from intermediate to essentially complete in the presence of 0.6% sodium chloride.

3. Higher concentrations of sodium chloride generally increased the degree of hemolysis produced by phenmetrazine hydrochloride solutions in the presence of 0.6% sodium chloride. In the case of 5.40% solutions of phenmetrazine hydrochloride, hemolysis was essentially complete in presence of 0.6, 0.9, 1.2, and 1.5% sodium chloride.

4. No appreciable discoloration of oxyhemoglobin resulted from the presence of 0.1% sodium carbonate. Triple distilled water, 0.1% sodium chloride, 0.1% sodium carbonate, 0.01% saponin, and a solution containing 0.1% sodium carbonate and 0.01% saponin caused the same degree of hemolysis within experimental error.

5. The slight time lapse between the addition of blood to the solution under study and the subsequent shaking of the mixture seemed to produce no appreciable variation in experimental data.

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Modification of Physical Properties of Certain Antitussive and Antihistaminic Agents by Formation of N-Cyclohexylsulfamate Salts

By JAMES A. CAMPBELL and JAMES G. SLATER

The N-cyclohexylsulfamic acid salts of four well known therapeutic agents were prepared. Salts of two of the compounds, dextromethorphan and chlorpheniramine, were found to have greatly improved taste and increased solubility. Accelerated aging studies indicate good stability.

PROMINENT among the factors which must be considered in the development of oral dosage forms are those of solubility and taste acceptability. These factors present no great problem provided the drug is to be administered in such dosage forms as capsules or tablets which are designed to be swallowed as a unit. However, in those cases where it is desirable to administer a drug in liquid form, chewable tablet, lozenge, or other such forms, solubility and taste acceptability become factors of prime consideration.

Received April 28, 1961, from the Pharmacy Research Laboratory, Miles Laboratories, Inc., Elkhart, Ind.
Accepted for publication August 18, 1961.
Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.

A crystalline precipitate appeared in solutions of several concentrations of butacaine sulfate in 1.2 and 1.5% sodium chloride before the addition of blood. In contrast to the effect produced on butacaine sulfate solutions by increased proportions of sodium chloride, several solutions of pramoxine hydrochloride in some concentrations of sodium chloride exhibited evidence of precipitation after incubation and centrifugation of the blood-salt mixtures. Higher concentrations of sodium chloride appeared to decrease the precipitation until the effect was eliminated as far as visual observation could detect.

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SUMMARY

1. An increase in the concentration of sodium chloride reduced the degree of hemolysis produced by procaine hydrochloride, 2-propoxyprocaine hydrochloride, and ammonium salicylate solutions in an environment of 0.6% sodium chloride. A similar effect was noted in most of the concentrations of ammonium benzoate studied.

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3. Higher concentrations of sodium chloride generally increased the degree of hemolysis produced by phenmetrazine hydrochloride solutions in the presence of 0.6% sodium chloride. In the case of 5.40% solutions of phenmetrazine hydrochloride, hemolysis was essentially complete in presence of 0.6, 0.9, 1.2, and 1.5% sodium chloride.

4. No appreciable discoloration of oxyhemoglobin resulted from the presence of 0.1% sodium carbonate. Triple distilled water, 0.1% sodium chloride, 0.1% sodium carbonate, 0.01% saponin, and a solution containing 0.1% sodium carbonate and 0.01% saponin caused the same degree of hemolysis within experimental error.

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By JAMES A. CAMPBELL and JAMES G. SLATER

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PROMINENT among the factors which must be considered in the development of oral dosage forms are those of solubility and taste acceptability. These factors present no great problem provided the drug is to be administered in such dosage forms as capsules or tablets which are designed to be swallowed as a unit. However, in those cases where it is desirable to administer a drug in liquid form, chewable tablet, lozenge, or other such forms, solubility and taste acceptability become factors of prime consideration.

Received April 28, 1961, from the Pharmacy Research Laboratory, Miles Laboratories, Inc., Elkhart, Ind.
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Some drugs present no taste problem because most people do not find the characteristic taste objectionable. Many drugs, even though moderately distasteful, are acceptable to some people because they are therapeutic agents and are not intended to appeal to the gustatory sense. However, it appears that people, in general, have come to expect and demand palatability in therapeutic agents.

Unpleasant tastes are overcome or improved upon in various ways. The usual method involves the judicious selection and use of additives. Cook (1) noted three factors in successful flavoring of pharmaceuticals: "(*a*) selection of a harmonious flavor, (*b*) effective masking, and (*c*) utilizing the undesirable taste as a base upon which a palatable formulation is built." Entrekin and Becker (2) found that the following would aid in disguising the bitter taste of quinine hydrochloride; adding citric acid, adding sodium chloride to some types of syrups, increasing sugar concentration, and increasing viscosity. In a recent survey of types of flavors used by pharmaceutical manufacturers for improving taste, Wesley (3) divided the flavors into two main categories: (*a*) fruit, and (*b*) nonfruit. It was observed that there was a trend toward the use of fruit flavors with a single flavor being used in 72% of the cases and with a combination being used in 28% of the cases. Pharmaceutical flavors (4) in descending order of frequency of use were found to be cherry, fruit (nonspecific), orange, raspberry, chocolate, and mint.

With the skillful application of masking and flavoring techniques available, one can usually produce an acceptable product provided there are no stringent restrictions on dilution. However, in cases where it is desirable to administer drugs in a rather concentrated form, such as drop dosage, the usual methods of taste control are sometimes inadequate. In such a situation, one must seek other ways of dealing with the taste problem while keeping in mind the solubility limitations.

Conceivably, one might be able to modify the taste characteristics of certain drugs by formation of salts or complexes of the drug which have more acceptable taste characteristics. This might be achieved in either of two ways; the formation of an insoluble compound which would be tasteless (5), or the formation of a soluble salt possessing an improved taste. One obvious disadvantage of the former would be the insolubility which would preclude its use as a solution. On the other hand, the formation of a soluble salt with improved taste is not always easy to achieve.

The objective in the study to be described was the formation of soluble, palatable, stable salts of certain characteristically bitter amine drugs. Four well-known therapeutic agents were chosen for the study: two antitussive agents—noscapine and dextromethorphan; and two antihistaminic agents—thenylpyramine and chlorpheniramine (6). The drugs, in the form of the free bases, were caused to react with N-cyclohexylsulfamic acid to form the corresponding salts.

A preliminary taste evaluation of the resulting salts was performed as follows: Aqueous solutions of the new salts as well as the commonly occurring salts were prepared in 0.004 *M* concentration. The solutions were tasted by a panel of six people. The dextromethorphan and chlorpheniramine salts of N-cyclohexylsulfamic acid were judged to be superior to the original salts (dextromethorphan hydrobromide and chlorpheniramine maleate) by the panel, whereas the new salts of noscapine and thenylpyramine were judged to have no advantage over the original forms. The latter two compounds were not considered further but the dextromethorphan N-cyclohexylsulfamate and chlorpheniramine N-cyclohexylsulfamate were subjected to further investigation.

EXPERIMENTAL

Formation of the Salts

Two different methods for forming the salts were utilized. These will be referred to as Methods A and B.

Method A.—Equivalent quantities of N-cyclohexylsulfamic acid and the free amine base were placed in a round-bottomed flask. Absolute ethanol was added in the proportion of 4 ml. of ethanol to each Gm. of N-cyclohexylsulfamic acid. This mixture was refluxed for a period of 1 hour and then concentrated to approximately one-half its original volume. Approximately three times its volume of ether was added to cool the solution. After precipitation was complete, the precipitate was collected on a filter and dried under vacuum.

Method B.—A slurry of the free base in water was titrated with a 0.1 *N* solution of N-cyclohexylsulfamic acid to the end point using a titrimeter. There was a gradual dissolution of the base as the end point was approached with a complete disappearance at the end point. The solution was evaporated to dryness using a Rinco evaporator at temperatures ranging up to 85°.

Dextromethorphan N-Cyclohexylsulfamate

The compound is a white granular substance. One gram is soluble in 9.7 ml. water. Melting characteristics are as follows: 80° (partial liquefaction), 217–218° (decompn).

Anal.—Calcd. for $C_{18}H_{25}NO \cdot C_6H_{13}NO_3S \cdot H_2O$, M. W. 468.66: N total, 5.99. Found: N total (Kjeldahl), 6.14.

Infrared Spectra.—Infrared spectra of equivalent concentrations of dextromethorphan N-cyclohexyl-

sulfamate and of an equimolar mixture of dextromethorphan and N-cyclohexylsulfamic acid were run in potassium bromide pellets. The spectra are shown in Fig. 1. Certain absorption peaks appeared in the spectrum of the compound but were absent in the spectrum of the reactants. These peaks were found at the following points: 760, 800, 1150, and 1170 cm.$^{-1}$. On the other hand, certain absorption peaks appeared in the spectrum of the mixture but were absent in the spectrum of the compound. These were observed at the following points: 705, 820, 1050, 1220, 1231, 1250, and 3250 cm.$^{-1}$.

Optical Rotation.—The compound was found to have the following optical rotation: $[\alpha]_D^{25} = +28°$ to $+29°$ (2.5% solution in water).

Stability Studies.—Aqueous solutions of the compound were prepared at pH levels of 4, 5, 6, and 7 using phthalate–NaOH and KH_2PO_4–NaOH buffer mixtures. After assay by optical rotation, the solutions were placed in an oven at 50°. The solutions were assayed and inspected at the end of 1 month and 6 months. The results are shown in Table I. From the data it appears that dextromethorphan N-cyclohexylsulfamate in aqueous solution has good stability when maintained at a pH not greater than 4.

TABLE I.—STABILITY OF DEXTROMETHORPHAN N-CYCLOHEXYLSULFAMATE IN AQUEOUS SOLUTION AT 50°

	Original	One Month		Six Months	
pH	% Found	% Found	Appearance	% Found	Appearance
4	2.14	2.13	No change	1.83	Some sediment
5	2.11	1.22	Little sediment	...	Sediment
6	2.09	0.74	Sediment	...	Sediment
7	2.10	0.58	Sediment	...	Sediment

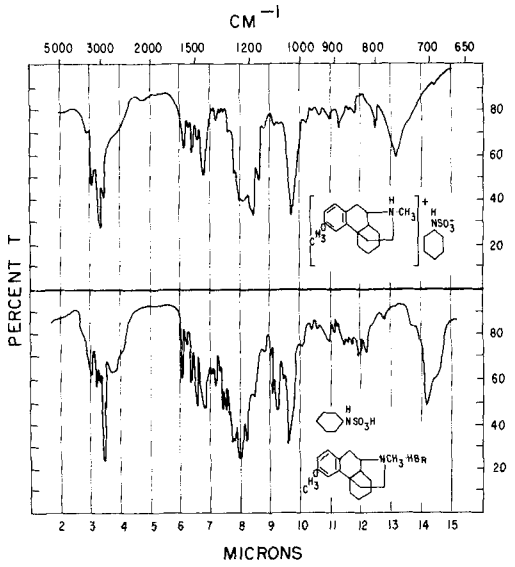


Fig. 1.—Infrared spectra of dextromethorphan N-cyclohexylsulfamate (upper section) and an equimolar mixture of dextromethorphan hydrobromide and N-cyclohexylsulfamic acid (lower section).

Chlorpheniramine N-Cyclohexylsulfamate

This compound appears as a white, or slightly pink crystalline substance. One gram is soluble in 10 Gm. of water. The melting point was found to be 90–95°. It was found to have an absorption maximum at 262 mμ.

Anal.—Calcd. for $C_{16}H_{19}ClN_2 \cdot C_6H_{13}NO_3S$, M.W. 454.04: N total, 9.25; Cl, 7.8. Found: N total (Kjeldahl), 9.27; Cl, 7.73.

Infrared Spectra.—Infrared spectra of equivalent concentrations of chlorpheniramine N-cyclohexylsulfamate and of an equimolar mixture of chlorpheniramine and N-cyclohexylsulfamic acid were run in potassium bromide pellets. The spectra are shown in Fig. 2. Certain absorption peaks appeared in the spectrum of the compound that were absent in the spectrum of the mixture. These peaks can be observed at 940, 1122, and 1550–1560 cm.$^{-1}$. Likewise, certain peaks were found in the spectrum of the mixture that were absent in the spectrum of the compound. These peaks can be observed at 725, 780, 830, 1200, 2700, and 3500 cm.$^{-1}$.

Stability Studies.—Aqueous solutions were prepared in a concentration of approximately 0.4%. The solutions were buffered at pH 4, 5, 6, and 7, respectively. The solutions were assayed by ultraviolet absorption at 262 mμ using a 0.4% chlorpheniramine maleate solution as the standard. After assay, the solutions were placed in an oven at 50°. The solutions were inspected and assayed at the end of 2 months. The results are shown in Table II. From the data contained in Table II it appears that chlorpheniramine N-cyclohexylsulfamate solution possesses good stability when maintained at a pH of 4.

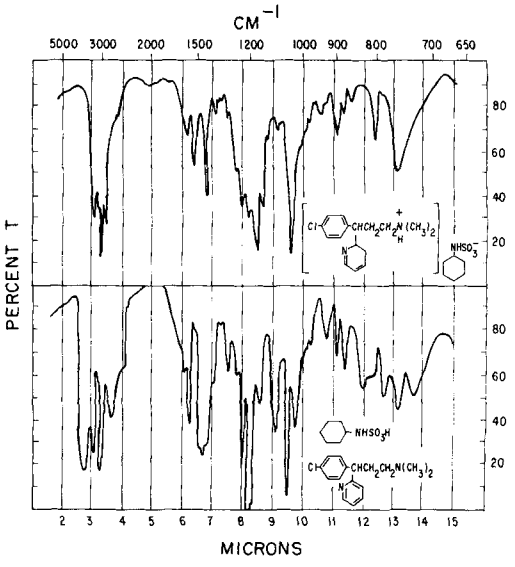


Fig. 2.—Infrared spectra of chlorpheniramine N-cyclohexylsulfamate (upper section) and an equimolar mixture of chlorpheniramine and N-cyclohexylsulfamic acid (lower section).

TABLE II —STABILITY OF CHLORPHENIRAMINE N-CYCLOHEXYLSULFAMATE IN AQUEOUS SOLUTION AT 50° FOR 2 MONTHS

pH	Original, mg./ml.	Two Months— mg./ml.	Appearance	% Loss
4	3.24	3.30	Clear	0
5	4.05	3.46	Clear	15
6	3.65	3.20	Clear	13
7	3.85	2.62	Sediment	32

SUMMARY AND CONCLUSIONS

1. The N-cyclohexylsulfamic acid salts of dextromethorphan and chlorpheniramine were prepared and characterized.

2. The salts were found to have greatly improved bitterness thresholds over that of the commonly occurring salts of the compounds.

3. When submitted to accelerated aging conditions, the compounds appeared to have good stability provided they were maintained at optimum pH levels.

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Influence of Kinetin and Gibberellic Acid on the Growth and Alkaloid Patterns in *Datura meteloides*

By DARRYL G. AMBROSE† and LEO A. SCIUCHETTI

Datura meteloides D.C. was administered four weekly-doses of 25 mcg. of kinetin (K) or gibberellic acid (GA). Different growth responses were induced by each treatment. Significant increases in height were noted in plants treated with GA whereas those treated with K were shorter than controls. Considerable increases in dry weights were noted in the GA-treated plants while reductions were observed in K-treated plants. The concentration of alkaloids in the organs of plants treated with GA was markedly reduced but no significant changes occurred with K. The total alkaloid content per plant was less in each of the treated groups but total stem alkaloids were greater in those treated with GA. The per cent of chlorophyll a and b was decreased by GA but increased by K.

PREVIOUS INVESTIGATIONS with kinetin, 6-(furfurylamino)-purine, and gibberellic acid (GA) indicate that these substances induce a profound effect on plant growth. Kinetin (K) was isolated and synthesized in 1955 by Miller, *et al.* (1, 2). This growth regulator has been shown to affect cell division (1, 3), cell enlargement as well as cell division (3, 4), root development (5, 6), shoot formation (7), and seed germination (8–10). Kinetin substituted for the light requirement of *Lemna minor* (11) and caused a modification of sporeling ontogeny in *Marsilea vestita* (12). K-treatment of lupin embryos *in vitro* caused about a twofold increase in alkaloid synthesis (13). Mothes, *et al.*, have shown that K affects nitrogen metabolism (14). Soluble nitrogen compounds in excised tobacco leaves were drawn from other leaf areas or added from the outside by K (14). A review of the literature has indicated that research has not been performed on the effects of K on the growth and alkaloid biogenesis in *Datura meteloides*.

The metabolic effects of GA on many plants (15–17), as well as on some medicinal plants (18), have been well established. The concentration of alkaloids in the plant organs was reduced and stem weight was increased in *Datura stramonium* (18–21), *Atropa belladonna* (19, 20), *Hyoscyamus niger* (22), and tobacco (23, 24). Increased height and variable results on leaftop, and root growth have been reported (18–24).

In view of the fact that K had not been employed on *Datura* species, it was decided to investigate the effect of this substance on the growth pattern, alkaloid formation, and chlorophyll content of *Datura meteloides*. It was further desired to ascertain whether this plant would respond to a GA treatment in a manner similar to that previously reported for *Datura stramonium*, and, also, to observe whether K and GA would induce similar metabolic effects.

Received January 8, 1962, from Oregon State University, School of Pharmacy, Corvallis.
Accepted for publication February 5, 1962.
Presented to Section Np, A.A.A.S., Denver meeting, December, 1961.
This work was supported by National Science Foundation grant G12131 for an U.R.P.P. program during the summer of 1960.
† Recipient of the 1961 Edwin Leigh Newcomb Award, 2nd place, undergraduate division.
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EXPERIMENTAL

Procedure.—*Datura meteloides* plants employed in this study were grown under greenhouse conditions. Seeds were germinated in flats containing a soil mixture of two parts of loam and one part of sand. Approximately 50 Gm. of complete organic fertilizer[1] was incorporated into the soil of each flat. On July 5, 1960, 72 14-day-old seedlings were transplanted into individual 1-gallon metal containers that had been filled with the sandy loam mixture. About 5 Gm. of complete fertilizer had been thoroughly mixed into the soil of each container prior to transplantation. The 72 seedlings were placed on eight different benches in the greenhouse. Nine plants were arranged on each bench by use of a randomization table (25). Nine plants each of *Datura tatula*, *Atropa belladonna*, and *Hyoscyamus niger* were also randomly arranged with *Datura meteloides* on each of the eight benches. The other species were being investigated simultaneously by other workers. Each species was labeled according to the following plan for treatment and harvest: three groups of 24 each were designated as controls, K-treated plants, and GA-treated plants; of the 24 plants within each group, eight each were harvested at 0, 2, and 4 weeks. The division of the plant into its morphological parts during each harvest, fresh and dry weight determinations, pulverization, and storage of the powdered material were conducted in a manner described in a previous publication (19).

On July 14 (zero time), the 16 plants for the K group and the corresponding plants of the GA group designated for the last two harvests were treated with a single 25-mcg. dose of the specific growth regulator. Treatments were continued hereafter at weekly intervals. The dose of the chemical was delivered from a micro pipet onto the surface of the youngest unfolding leaf by applying 0.1-ml. portions of the respective aqueous solutions. The GA[2] solution was prepared freshly from a stock alcoholic solution of GA by dilution with distilled water. The K solution was freshly prepared before each treatment by dissolving 2.5 mg. of kinetin in approximately 3 ml. of a stock potassium hydroxide solution having a pH of 11.2. This solution was back titrated with dilute sulfuric acid ($10^{-3}N$) to a pH of 6.5. Then, enough distilled water was added to make 10 ml. At this time, initial height measurements were made on all plants and the first harvest of eight plants from each group was made. Height measurements were taken weekly thereafter.

Growth Effects.—The K-treated plants and the controls resembled each other in habit and were more bushy than the GA-treated plants (Fig. 1). At the final harvest, some of the plants treated with K appeared darker in color than the controls. Typical gibberellin-effects were noted in the GA-treated group, such as greater internodal elongation, increased height, and a spindlier appearance (Fig. 1). The leaves of the GA-treated plants appeared slightly chlorotic initially, whereas the stems demonstrated an apparent chlorosis at the final harvest.

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. *Anal.*—5% total nitrogen, 3% available phosphate, 2% available potash.

[2] The GA employed in this study was furnished through the courtesy of Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Ind.

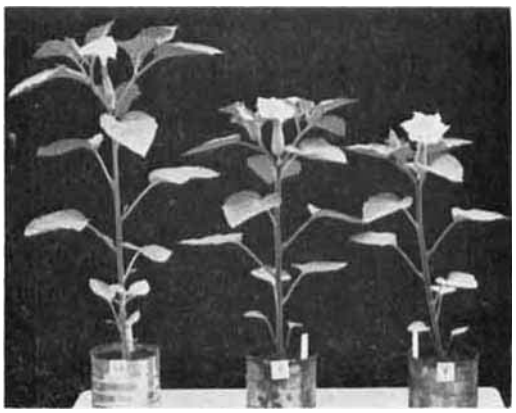


Fig. 1.—Effect of K and GA treatments on the growth of *Datura meteloides*. GA., Gibberellic acid; C, control; K, kinetin. Plants at the final harvest were about 7 weeks old.

Height measurements indicated that plants treated with K were shorter than controls throughout the observation period while the GA-treated plants grew progressively taller (Fig. 2). Treatment with GA induced height increases greater than 40% from the second through fourth weeks.

Fresh and Dry Weights.—The treatment with K caused less growth at the final harvest, as indicated by fresh and dry weight data (Table I). The final dry weights were about 86, 80, and 96% that of controls for leaves-tops, stems, and roots, respectively (Table I). The decreased growth of this group became apparent between the second and fourth weeks. On the other hand, a general favorable response on growth was induced by treatment with GA. The total dry weight per plant of this group attained its maximum gain (about 15%) at the last harvest (Table I). The largest increase in dry weight was noted in the stems. About a 40%

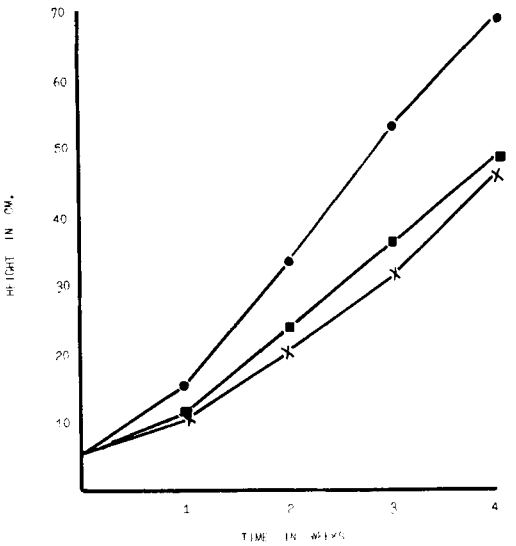


Fig. 2.—Height measurements of *Datura* plants. ■—■, Controls; ×—×, 25 mcg. kinetin; ●—●, 25 mcg. GA.

TABLE I.—WEIGHTS OF *Datura* PLANT PARTS (AV./PLANT/GROUP)

Treatment and Harvest Time, wk.	Total Weight			Leaves-Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control, 0	1.12	0.16	...	0.83	0.12	...	0.14	0.02	...	0.15	0.02	...
Kinetin, 0[a]	1.19	0.16	103.2[b]	0.86	0.12	102.6[b]	0.16	0.02	110.5[b]	0.17	0.02	100.0
GA, 0[a]	1.21	0.16	98.1[b]	0.87	0.11	97.4[b]	0.15	0.02	100.0[b]	0.19	0.02	100.0
Control, 2	37.23	4.01	...	20.36	2.59	...	7.76	0.71	...	9.11	0.71	...
Kinetin, 2	39.11	4.13	103.0	21.52	2.68	103.7	8.13	0.70	97.8	9.46	0.75	105.8
GA, 2	39.34	4.46	111.4	19.56	2.68	103.7	10.93	0.99	139.3	8.85	0.79	111.3
Control, 4	98.77	14.07	...	43.95	6.83	...	29.19	4.52	...	25.63	2.72	...
Kinetin, 4	87.15	12.08	85.9	38.38	5.87	86.0	24.05	3.60	79.7	24.72	2.61	95.8
GA, 4	108.26	16.15	114.8	47.09	7.25	106.2	37.37	6.35	140.4	23.80	2.56	93.8

[a] These plants had no treatment. [b] Figures obtained by calculations from weights taken in mg., tabulated data are rounded at second decimal place.

TABLE II.—TOTAL ALKALOID CONTENT[a] (MG.) OF *Datura meteloides*

Treatment and Harvest Time, wk.	Per Plant		Leaves-Tops		Stems		Roots	
	Total Alkaloids	% of Controls	Total Alkaloids	% of Controls	Total Alkaloids	% of Controls	Total Alkaloids	% of Controls
Control, 0	0.32	...	0.15	...	0.10	...	0.07	...
Control, 2	8.73	...	4.40	...	2.28	...	2.05	...
Kinetin, 2	8.43	96.6	4.03	91.6	2.23	97.8	2.17	105.9
GA, 2	7.76	88.9	3.49	79.3	2.38	104.4	1.89	92.2
Control, 4	31.30	...	10.92	...	10.85	...	9.53	...
Kinetin, 4	26.85	85.8	8.81	80.7	8.64	79.6	9.40	98.6
GA, 4	28.43	90.8	8.70	79.7	12.06	112.0	7.67	85.5

[a] Calculated from dry weight and alkaloid analyses data; per plant = leaves-tops + stems + roots.

TABLE III.—CHLOROPHYLL A AND B CONTENT OF *Datura* AERIAL PARTS

Treatment	Leaves-Tops				Stems			
	2 Weeks		4 Weeks		2 Weeks		4 Weeks	
	Per cent	Control, %	Per cent	Control, %	Per cent	Control, %	Per cent	Control, %
				Chlorophyll a				
Control	0.538	...	0.223	...	0.037	...	0.013	...
Kinetin	0.545	101.3	0.255	114.3	0.040	108.1	0.016	123.1
GA	0.473	87.9	0.220	98.7	0.034	91.9	0.012	92.3
				Chlorophyll b				
Control	0.420	...	0.163	...	0.035	...	0.014	...
Kinetin	0.410	97.6	0.198	121.5	0.042	120.0	0.015	107.1
GA	0.348	82.9	0.158	96.9	0.035	100.0	0.010	71.4

increase in stem weight was noted at the second and fourth weeks. The leaves-tops gained up to 15% in weight during the same period. Only at the final harvest was root dry weight less than controls.

Analysis for Alkaloids.—The alkaloid concentration, calculated as scopolamine, was determined by the Brummett-Sciuchetti method (20). The concentration of alkaloids (mg./Gm.) in the organs of the K-treated plants approximated that of the controls, while the concentration in the GA-treated plants was considerably lower in all plant parts throughout the stages of growth (Fig. 3.). The reductions of about 25% in the leaves-tops and the stems at the second and fourth weeks were considered significant.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant part by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table II). The total alkaloid content generally was markedly reduced in the leaves-tops and stems by the K treatment. The reductions, compared with controls, amounted to about 20% at the final harvest. This was due mainly to decreased growth of these organs. No significant changes occurred in the roots of K-treated plants. Treatment with GA caused about a 20% reduction in the alkaloid content of the leaves-tops at each harvest and about a 15% decrease in the roots at the final harvest. Due to increased stem growth, the GA-treated plants showed gains in total stem alkaloids at each harvest (Table II).

Chlorophyll Content.—The chlorophyll analysis was conducted by a method described by Gjerstad (26). His method was modified by employing dried material, 1-Gm. samples of leaves-tops and 5 Gm. of stems, and using a Soxhlet extraction apparatus. The chlorophyll was completely extracted from the powdered material with a 20% water in acetone solvent. The results of the chlorophyll analysis are shown in Table III. The per cent of chlorophyll a and b in the leaves-tops and stems of the K-treated plants was markedly increased whereas that of the GA-treated plants was reduced considerably (Table III).

DISCUSSION AND CONCLUSIONS

Specific metabolic effects were noted in the plants treated with kinetin. Although the K-treated plants generally appeared similar to the controls in habit, several differences were noted. The treated plants were shorter than controls throughout the observation period and the leaves appeared darker at the final harvest. Growth of the aerial parts was retarded, but this did not occur until a period of time between the second and fourth weeks. The fresh and dry weights of the aerial parts were reduced up to 20% that of the controls at the final harvest. These observations indicate that there was a delayed response to the kinetin. It is altogether possible that a significant reduction in weight would have occurred had this experiment been allowed to continue for a longer period of time. The concentration of alkaloids was not appreciably altered in the plant parts by the K-treatment. The decreased alkaloid content per plant, per leaves-tops, and per stem at the final harvest was due primarily to the adverse effect of kinetin on growth. A most interesting aspect observed from the K-treatment was an increase in the per cent of chlorophyll in the aerial parts. The greatest increase was at the final harvest. This confirms the observations that the leaves generally appeared darker green in color at the final harvest. The fact that the increased per cent of chlorophyll generally directly paralleled the decrease in plant growth, as shown by the dry weight data, might indicate that there was not an increased capacity for chlorophyll synthesis by the K-treated plants; instead, the higher per cent of chlorophyll was probably due to concentration. Future histological work should clarify this phenomenon.

Datura meteloides responded as do other solanaceous plants when treated with GA (18–22). The following characteristic gibberellin-effects were noted: taller and spindlier plants, greater internodal elongation, slight chlorosis of the leaves and stems, significantly increased stem growth, and decreased concentration of alkaloids. Growth was stimulated by GA since increases in dry weights were generally noted throughout the observation period. In spite of a favorable effect on growth, the decreased concentration of alkaloids in the organs resulted in about a 10% reduction in total alkaloid content per plant at the final harvest. The reduced chlorophyll per cent in the aerial parts of the treated plants confirms our observations which were reported previously that GA-treated plants appeared chlorotic (18–20). The decreased chlorophyll per cent in the GA-treated plants is also in agreement with that reported for spearmint (27), but is not in agreement for that reported for peppermint (26) and foxglove (28). In this experiment, as well as the one reported for spearmint (27), the decrease in chlorophyll per cent was accompanied by appreciable increases in growth. This would suggest that the apparent paleness of leaves from gibberellin treatment is most likely due to dilution rather than a direct effect on chlorophyll synthesis.

Kinetin generally induced a different effect than did GA. The following differences were noted (K *vs.* GA): shorter and bushier plants *vs.* taller and spindly plants; decreased growth *vs.* increased growth; no change in alkaloid concentration in the plant organs compared with controls *vs.* appreciable decreases; a delayed response following treatment *vs.* a rapid response. These differences suggest that K and GA possess different modes of action in various metabolic processes involved in plant growth and biosynthesis.

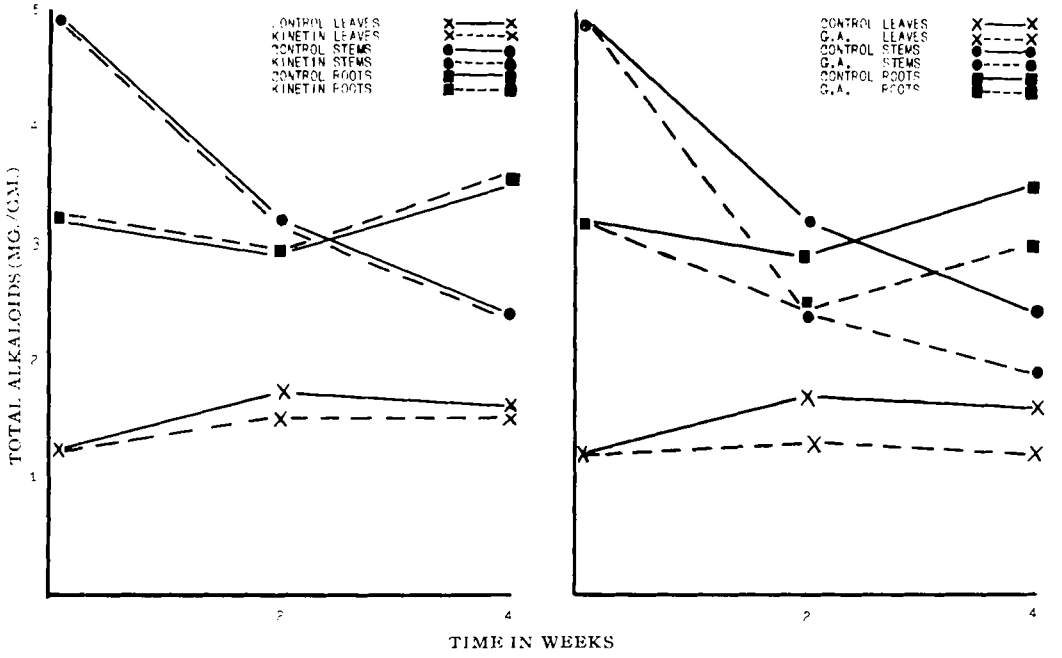


Fig. 3.—Alkaloid pattern of kinetin and gibberellic acid-treated plants. Left, K-treated; right, GA-treated.

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Separation and Identification of Sympathomimetic Amines by Gas-Liquid Chromatography

By E. BROCHMANN-HANSSEN and A. BAERHEIM SVENDSEN

A number of commonly used sympathomimetic amines have been subjected to gas chromatographic analysis on low-loaded columns. With the exception of certain isomers, most compounds can be separated on a column of silicone rubber SE-30. Many of the amines investigated react with ketones and the products produce sharp, symmetrical peaks on the gas chromatogram. Ephedrine and pseudoephedrine can be separated and identified on the basis of the difference in the rate of reaction with acetone. Monohydroxyphenols show strong adsorptive effects as the free bases but can readily be gas chromatographed as the acetone derivatives. The dihydroxyphenols are converted to the triacetyl derivatives which are treated with hexamethyldisilazane prior to the gas chromatographic analysis.

OF THE HUNDREDS of sympathomimetic amines which have been synthesized or isolated from natural sources, about 25 find widespread use in medical practice (1). Many methods for their identification have appeared in the literature (2–9). The official products are usually identified by color reactions, precipitation reactions, or on the basis of the melting ranges of the free amines, their salts, or prepared derivatives. Although these methods often lack specificity, they are usually satisfactory when the problem is to confirm the identity of a single compound which is available in sufficient amount. When mixtures of several amines are involved, as is often the case in pharmaceutical preparations, a separation is usually necessary before the components can be identified. In biological work, the problem is further complicated by the limited amounts available and by the presence of amines which are normal products of the animal body or formed by decomposition of biological material. Another complication stems from the fact that the metabolic products of certain sympathomimetic amines are themselves used as drugs (10, 11).

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EXPERIMENTAL

A Barber Colman model 15 gas chromatograph equipped with an argon β-ionization detector (radium 226) was used for the experimental work. The colums were glass U-tubes, 6 to 8 feet in length and having an inner diameter of 3 mm. The solid support materials were Gas-Chrom P, 100 to 140 mesh, and Chromosorb W, 60 to 80 mesh, both of which were washed with concentrated hydrochloric acid and methanolic potassium hydroxide and treated with hexamethyldisilazane (14). The stationary phases were applied by means of a solution in toluene or butanone as described by Horning, *et al.*, (15). The amines were introduced with a Hamilton microliter syringe as 1.0 μl. of a 0.5 to 1.0% solution of the free bases in chloroform, acetone, or butanone. A number of the amines were found to

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Separation and Identification of Sympathomimetic Amines by Gas-Liquid Chromatography

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EXPERIMENTAL

A Barber Colman model 15 gas chromatograph equipped with an argon β-ionization detector (radium 226) was used for the experimental work. The colums were glass U-tubes, 6 to 8 feet in length and having an inner diameter of 3 mm. The solid support materials were Gas-Chrom P, 100 to 140 mesh, and Chromosorb W, 60 to 80 mesh, both of which were washed with concentrated hydrochloric acid and methanolic potassium hydroxide and treated with hexamethyldisilazane (14). The stationary phases were applied by means of a solution in toluene or butanone as described by Horning, *et al.*, (15). The amines were introduced with a Hamilton microliter syringe as 1.0 μl. of a 0.5 to 1.0% solution of the free bases in chloroform, acetone, or butanone. A number of the amines were found to

react with the ketone solvents to produce reaction products which gave sharp, symmetric peaks on the chromatograms. The catechol amines, epinephrine and levarterenol, were first converted to the triacetyl derivatives (16) and then treated with an excess of hexamethyldisilazane in tetrahydrofuran, either at room temperature overnight or by refluxing for about 2 to 3 hours.

RESULTS AND DISCUSSION

The retention values of 16 sympathomimetic amines and/or their derivatives are listed in Table I. Ephedrine and pseudoephedrine do not separate as the free bases on silicone rubber SE-30, Fig. 1. However, pseudoephedrine reacts relatively rapidly

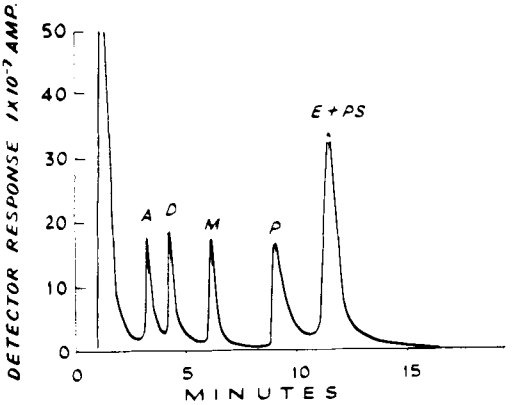


Fig. 1.—Gas chromatogram of six sympathomimetic amines on silicone rubber SE-30, 1.15%. A, amphetamine; D, methamphetamine; M, mephentermine; P, phenylpropanolamine; E, ephedrine; and PS, pseudoephedrine. Operating conditions; *cf.* Table I, footnotes[a], [c].

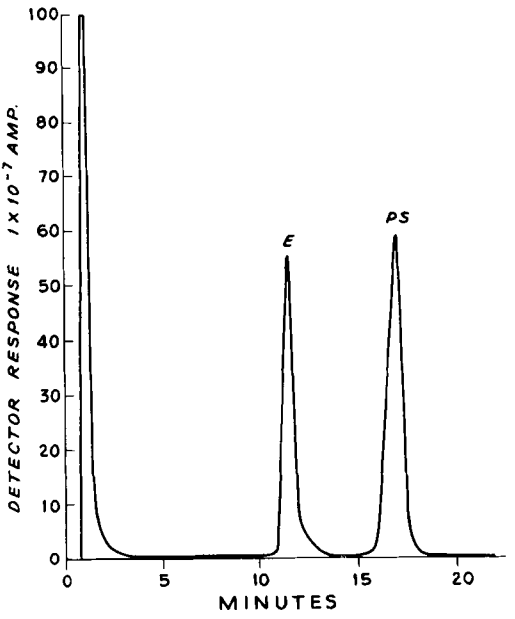


Fig. 2.—Gas chromatogram of an acetone solution of ephedrine (E) and pseudoephedrine (PS) after 3 hours at room temperature. Operating conditions, *cf.* Table I, footnotes[a], [c].

with acetone, while the reaction between acetone and ephedrine is very slow and incomplete. The reaction rate was studied gas chromatographically on the basis of the peak areas corresponding to the free amine and its ketone derivative. The relative rates of reaction of ephedrine and pseudoephedrine are expressed semiquantitatively in Table II. The two amines could easily be separated and/or identified by preparing an acetone solution and allowing it to stand for 2 to 3 hours prior to the gas chromatographic analysis, Fig. 2. Whereas all

TABLE I.—RETENTION TIMES OF SYMPATHOMIMETIC AMINES ON SILICONE RUBBER SE-30, 1.15%[a]

Compound	Column Temp., °C.	Retention Time, min. Base	Acetone Deriv.	Butanone Deriv.
Amphetamine	82[b]	7.2	16.9	28.5
Cyclopentamine		5.6	...	...
Mephentermine		16.7	...	...
Methamphetamine		10.6	...	...
Propylhexedrine		10.3	...	...
Tuaminoheptane		1.9	4.9	8.0
Amphetamine	104[c]	3.3	6.2	9.5
Cyclopentamine		2.6	...	...
Ephedrine		11.4	17.6	30.0
Mephentermine		6.1	...	...
Methamphetamine		4.6	...	...
Phenylpropanolamine		9.1	13.4	23.5
Propylhexedrine		4.0	...	...
Pseudoephedrine		11.6	16.4	27.0
Tuaminoheptane		with solvent	2.3	3.3
Benzphetamine	135[d]	15.0	...	...
Hydroxyamphetamine		2.9	5.0	7.0
Metaraminol		...[f]	9.5	14.0
Phenylephrine		9.0[g]	9.5	13.7
Synephrine		9.1[g]	9.5	14.2
Epinephrine[h]	179[e]	13.4	...	...
Levarterenol[h]		12.4	...	...

[a] Column length, 8 ft.; i.d., 3 mm.; solid support, Gas-Chrom P, 100 to 140 mesh. [b] Inlet pressure, 25 lb. p.s.i.; flow rate, 33 ml./min. [c] Inlet pressure, 21.5 lb. p.s.i.; flow rate, 22.5 ml./min. [d] Inlet pressure, 31 lb. p.s.i.; flow rate, 30 ml./min. [e] Inlet pressure, 30 lb. p.s.i.; flow rate, 25 ml./min. [f] No elution peak observed. [g] Adsorptive effects caused a badly tailing peak; retention time dependent on sample size. [h] Triacetyl trimethylsilyl ether.

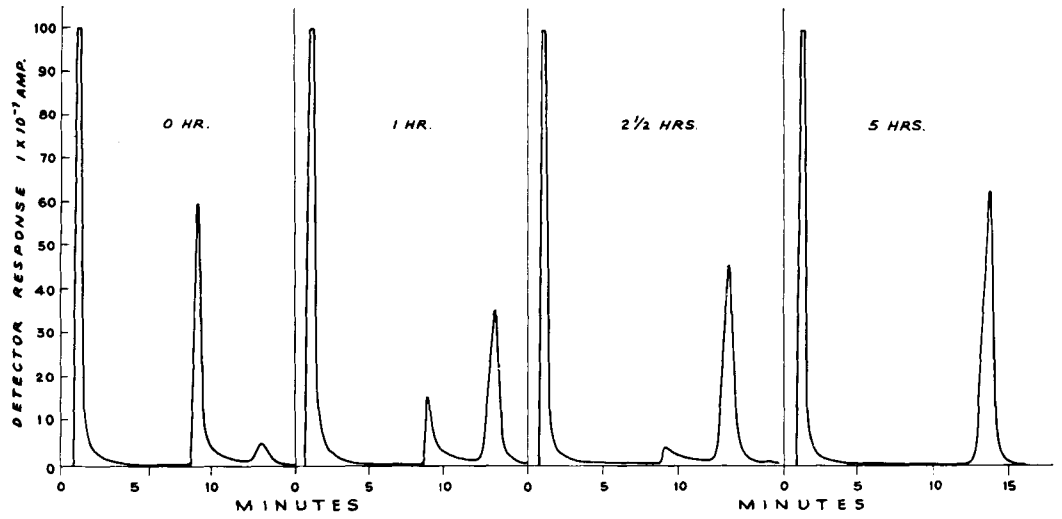


Fig. 3.—Gas chromatograms of an acetone solution of phenylpropanolamine. Formation of acetone derivative. *cf.* Table I, footnotes[a, c].

of the primary amines investigated reacted with ketones, this was not the case with all of the secondary amines. The rate of the reaction between phenylpropanolamine and acetone is illustrated in Fig. 3. As would be expected, benzphetamine, being a tertiary amine, gave no reaction with ketones. The reaction between ketones and certain primary amines has been studied by Bergel and Lewis (17, 18). According to these authors, the reaction involves an addition followed by loss of water to form an azomethine. This is accompanied by a dramatic increase in the specific rotation of optically active amines and amino acids. With secondary amines no such loss of water can occur; the reaction is probably a simple addition forming an amino alcohol. Butanone, because of the bulkier ethyl group, reacted more slowly than acetone. Methamphetamine and propylhexedrine had retention times that were very close and could not be separated on the silicone rubber column, but they were separated without difficulty on the more selective fluorosilicone fluid, Fig. 4.

Hydroxyamphetamine could easily be separated from the nonphenolic as well as from the other phenolic amines. Metaraminol could be distinguished from phenylephrine and synephrine on fluorosilicone fluid, but not on silicone rubber, Tables I and III. The isomeric phenylephrine and synephrine could not be separated on either stationary phase.

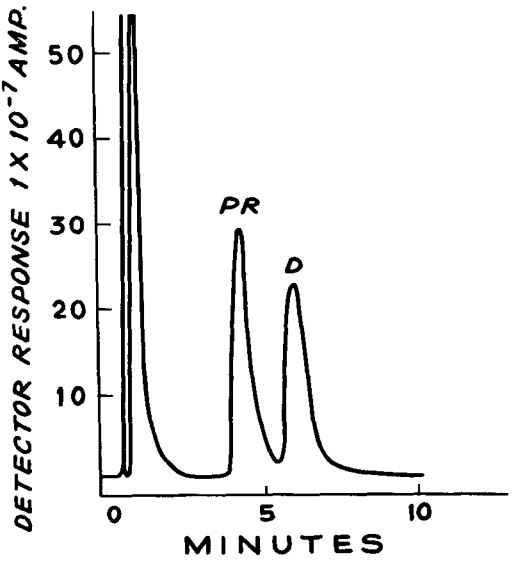


Fig. 4.—Gas chromatographic separation of propylhexedrine (PR) and methamphetamine (D). Stationary liquid, fluorosilicone fluid QF 1-0065, 2.8% on Chromosorb W, 60–80 mesh. Temp. 82°; inlet pressure, 20 lb. p.s.i.; flow rate 50 ml./min. Column, 6 ft. in length, 3 mm. i.d.

Table II.—Reaction of Ephedrine and Pseudoephedrine with Acetone as a Function of Time[a]

Reaction Time, hr.	Ephedrine 22° Eph.	Eph./Ac.	Ephedrine 56–57° Eph.	Eph./Ac.	Pseudoephedrine 22° Ps.E.	Ps.E./Ac.	Pseudoephedrine 56–57° Ps.E.	Ps.E./Ac.
0	++++	−	++++	...	++++	−	++++	...
1/4	++++	−	++++	Trace	+++	+	++	++
1	++++	−	+++	+	++	++	+	+++
2	++++	−	+++	+	+	+++	Trace	++++
5	++++	−	++	++	Trace	++++	−	++++
10	++++	−	+	+++	−	++++	...	...
24	+++	+	...	...				

[a] Ten milligrams of amine was dissolved in 1 ml. of acetone. Heating at 56–57° was carried out under reflux in micro equipment. The solutions were gas chromatographed under conditions described in Table I, footnotes[a, c].

The catechol amines are rather insoluble in most organic solvents and are, furthermore, firmly adsorbed on the low-loaded columns used in this work. Epinephrine and levarterenol were, therefore, converted to the triacetyl derivatives by treatment of an aqueous solution with acetic anhydride and sodium bicarbonate (16). Gas chromatographic analysis of triacetylepinephrine produced three peaks indicating that decomposition had taken place. This decomposition was probably due to the free alcoholic hydroxyl group which in the flash heater caused alcoholysis and transesterification resulting in a mixture of diacetyl-, triacetyl-, and tetraacetylepinephrine. Similar decomposition reactions have been observed by gas chromatography of acetylated morphine (19). Attempts to acetylate epinephrine completely did not entirely eliminate this problem. However, if the secondary alcohol group of triacetylepinephrine was converted to a trimethylsilyl ether, the resulting product gave a single, symmetrical peak. Levarterenol, after acetylation and treatment with hexamethyldisilazane, could be differentiated from epinephrine on the basis of the retention time, Table I.

TABLE III.—RETENTION TIMES OF SOME PHENOLIC AMINES ON FLUOROSILICONE FLUID, QF 1-0065[a]

Compound	—Retention Time, min.— Base[b]	Acetone Deriv.
Metaraminol	...	6.7
Phenylephrine	...	5.7
Synephrine	...	5.7

[a] Column, 6 ft. × 3 mm. i.d.; 2.8% QF 1-0065 (Dow Corning) on Chromosorb, 60–80 mesh; column temp., 138°; inlet pressure 30 lb. p.s.i. [b] No elution peaks were observed for the free amines.

CONCLUSION

With the exception of phenylephrine and synephrine, all the sympathomimetic amines studied could be separated and identified. The reaction with ketones was very useful as a means of separating and identifying certain amines which followed each other very closely as the free bases. The ketone derivatives, furthermore, gave nontailing peaks that should be suitable for quantitative work. Certain phenolic amines were difficult to gas chromatograph on low-loaded columns because of adsorptive effects. The monophenolic amines investigated gave good chromatograms as the ketone derivatives. The catechol amines gave best results by acetylation and treatment with hexamethyldisilazane prior to the gas chromatographic analysis.

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Synthesis of Diphenic Acid Derivatives

By WILLIAM D. ROLL† and GUSTAV E. CWALINA

Five new derivatives of diphenic acid were prepared for the purpose of studying their antibacterial, antihyperglycemic, antispasmodic, and/or local anesthetic activity: *o,o'*-bis(2-nitro-1,3-dihydroxypropyl)biphenyl disodium salt; *o,o'*-bis(3-benzoxy-2-benzamido-1-hydroxypropyl)biphenyl; *o,o'*-bis(β-dichloroacetamidoethyl)diphenate; N,N'-bis(benzenesulfonyl)-*o,o'*-diphenoylurea, and *m,m'*-bis(N-carboxymethyl)diphenamide.

VARIOUS types of derivatives of diphenic acid were synthesized for the purpose of making them available for pharmacological testing as possible antibacterial, antihyperglycemic, antispasmodic, and/or local anesthetic agents.

Roberts and Johnson (1) reported that diethylaminoethyl diphenate and diethylaminoethyl imidoester of diphenanilide possessed local anesthetic activity. Case and Koft (2) synthesized a similar compound, diethylaminoethyl 5,5-diaminodiphenate, which had marked local anesthetic activity. A number of substituted amine esters and amides of diphenic acid were prepared by Demers and Jenkins (3) for evaluation as possible antispasmodics and antihistamines. Compound VII of our derivatives contains an amide-ester linkage.

Woolley (4) has suggested that analogs of phenylalanine might function as antimetabolites

Received April 25, 1958, from Purdue University, School of Pharmacy, Lafayette, Ind.
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Roberts and Johnson (1) reported that diethylaminoethyl diphenate and diethylaminoethyl imidoester of diphenanilide possessed local anesthetic activity. Case and Koft (2) synthesized a similar compound, diethylaminoethyl 5,5-diaminodiphenate, which had marked local anesthetic activity. A number of substituted amine esters and amides of diphenic acid were prepared by Demers and Jenkins (3) for evaluation as possible antispasmodics and antihistamines. Compound VII of our derivatives contains an amide-ester linkage.

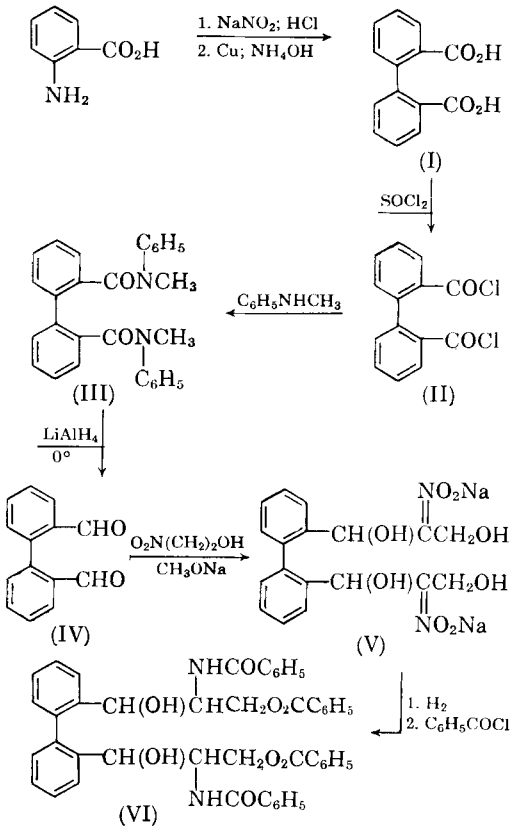
Woolley (4) has suggested that analogs of phenylalanine might function as antimetabolites

Received April 25, 1958, from Purdue University, School of Pharmacy, Lafayette, Ind.
Accepted for publication March 22, 1962.
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by interfering with the enzymatic transformations which are undergone by an essential metabolite probably related to phenylalanine. Such analogs might inhibit the incorporation of phenylalanine into protein. Structural analogs of the amino acid glycine might also serve as specific inhibitors of protein synthesis. Such compounds may have antibacterial activity since protein synthesis is a process fundamental to the growth of living organisms. Compounds V and VI can be regarded as analogs of phenylalanine, differing from the amino acid in four positions. Compound XI is an amide derivative of glycine. The report (5) of the oral antidiabetic compound tolbutamide[1] stimulated the synthesis of the sulfonylurea derivative of diphenic acid. Compound VIII has structural features similar to tolbutamide.

The synthetic procedure used for the preparation of these compounds may be briefly outlined as follows: *o,o'*-diphenic acid, I, synthesized by the procedure described by Atkinson and Lawler (6), was converted to *o,o'*-diphenoyl chloride, II, (7). In order to introduce an alkanol side chain, the acyl chloride was converted in a step-wise manner to *o,o'*-bis(N-methyl)diphenanilide, III, (8), *o,o'*-diphenaldehyde, IV, (8) and *o,o'*-bis(2-nitro - 1,3 - dihydroxypropyl)biphenyl disodium salt, V. The nitroalcohol was reduced with hydrogen and palladous oxide and isolated as the amide-ester *o,o'*-bis(3-benzoxy-2-benzamido-1-hydroxypropyl)biphenyl, VI.

One ester of diphenic acid was prepared by treating II with β-dichloroacetamidoethanol to form *o,o'*-bis(β-dichloroacetamido-ethyl)diphenate, VII. Two amides of diphenic acid, N,N'-bis(benzenesulfonyl) - *o,o'* - diphenoylurea, VIII, and *m,m'*-bis(N-carboxymethyl)diphenamide, XI, were synthesized by reacting II with benzenesulfonylurea (9) and *m,m'*-diphenic acid, X, with thionyl chloride and glycine.



EXPERIMENTAL

The sequence of synthetic reactions is shown by the diagrams in the text.

***o,o'*-Diphenic Acid, I.**—The procedure used for the synthesis of diphenic acid was that described in "Organic Syntheses" (6).

***o,o'*-Diphenoyl Chloride, II.**—This known compound was prepared from I and thionyl chloride. Yields varied from 89–98% and the product melted at 94–95° (7).

***o,o'*-Bis(N-methyl)diphenanilide, III.**—N-Methylaniline, 30.9 Gm. (0.29 mole), dissolved in 150 ml. of dry benzene, was added in 5-ml. portions over a 1-hr. period to a well stirred solution of 20 Gm. (0.072 mole) of II in 200 ml. of dry benzene. The reaction mixture was stirred for an additional 15 minutes after all of the amine had been added. The reaction mixture, after filtration, was washed with 3 *N* hydrochloric acid until the aqueous phase was colorless and then with distilled water until the aqueous phase was no longer acidic to litmus. The benzene layer was dried over anhydrous sodium sulfate for 24 hours, filtered, and the solvent removed *in vacuo*. The crude residue was recrystallized from acetone-water. The average yield of product (four runs) was 24.2 Gm. (80%); m.p. 160–161° (8).

***o,o'*-Diphenaldehyde, IV.**—In a 1-L. three-neck flask equipped with a mercury-sealed stirrer, a reflux condenser equipped with a calcium chloride tube, and dropping funnel was placed a solution containing 50 Gm. (0.119 mole) of III in 450 ml. of anhydrous tetrahydrofuran. This solution was cooled to 0° and a previously prepared solution containing 2.5 Gm. (0.066 mole) of lithium aluminum hydride in 65 ml. of anhydrous ether was placed in the dropping funnel. The hydride was added dropwise with rapid stirring over a 3-hr. interval. Stirring was continued for 5 additional hours while maintaining the temperature of the reaction mixture at 0°. At the end of this period, the cooling bath was removed and the excess lithium aluminum hydride was decomposed with an ice-cold solution of 5% hydrochloric acid. The reaction mixture was filtered into a separator and extracted with 100 ml. of ether. The ether solution was then washed successively with water, 5% hydrochloric acid, and water. The ether layer was dried over anhydrous sodium sulfate for 24 hours, filtered, and the solvent removed *in vacuo*. The residue weighed 20 Gm.

[1] Marketed as Orinase by The Upjohn Co.

The 2,4-dinitrophenylhydrazone of a portion of the residue was prepared; m.p. 168–169°. The reported (8) melting point is 168°.

***o,o'*-Bis (2-nitro-1,3-dihydroxypropyl)biphenyl Disodium Salt, V.**—In a 300-ml. round-bottom flask equipped with a mercury-sealed stirrer and 125-ml. dropping funnel, was placed a solution containing 20.0 Gm. (0.095 mole) of IV in 75 ml. of absolute methanol and 18.6 Gm. (0.204 mole) of 2-nitroethanol. The mixture was stirred and cooled to −10°. A freshly prepared solution of sodium methoxide containing 4.4 Gm. (0.190 mole) of sodium in 45 ml. of absolute methanol was cooled to −10° and transferred to the dropping funnel. The base was added in a dropwise manner over 30 minutes. The reaction mixture was stirred for 1 hour after all of the base had been added. The product was filtered, washed first with anhydrous ether, and then with three 10-ml. portions of cold absolute methanol. A yield of 36.5 Gm. (88%) was obtained. A portion of the product was recrystallized from a cold solution of acetone and water and analyzed for sodium (10).

Anal.—Calcd. for $C_{18}H_{18}N_2Na_2O_8$: Na, 10.55. Found: Na, 10.55, 10.56.

***o,o'* - Bis(3 - benzoxy - 2 - benzamido - 1 - hydroxypropyl)biphenyl, VI.**—A 35-Gm. quantity of *o,o'*-bis(2-nitro-1,3-dihydroxypropyl)biphenyl disodium salt, V, was dissolved in 120 ml. of glacial acetic acid in a Parr reduction bottle. Palladous oxide, 1 Gm., was added and the mixture was reduced at 50 p.s.i. for 24 hours. The reaction mixture was filtered and the filtrate heated *in vacuo* at 40°. The residue was dissolved in 250 ml. of water and extracted with ether. The aqueous phase was made strongly alkaline with sodium hydroxide.

A portion of the strongly basic solution was shaken with 45 Gm. of benzoyl chloride and 100 ml. of chloroform for 20 minutes in a separator. The mixture was allowed to stand for 12 hours. The chloroform layer was drawn off and separated. A white, gummy residue remained which, when stirred repeatedly with fresh portions of dry petroleum ether, solidified to a white crystalline solid. The crude product was recrystallized from ethanol-water yielding 10.5 Gm.; m.p. 85–87°.

Anal.—Calcd. for $C_{46}H_{40}N_2O_8$: N, 3.74; sapon. equiv., 187. Found: N, 3.80, 3.81; sapon. equiv., 173.

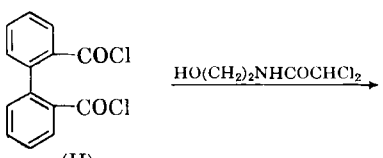
***o,o'* - Bis(β - dichloroacetamidoethyl)diphenate, VII.**—In a 250-ml. Erlenmeyer flask was placed 21.45 Gm. (0.15 mole) of methyl dichloroacetate. To this, with constant shaking, was added 9.15 Gm. (0.15 mole) of β-ethanolamine. The reaction mixture became warm and the heretofore colorless solution became bright red. When it had cooled to room temperature it was transferred to a 400-ml. beaker and allowed to crystallize at room temperature. The product, β-dichloroacetamidoethanol, was filtered and recrystallized from chloroform. It weighed 24.5 Gm. (95%); m.p. 85–86°.

To a cold (0°) well-stirred mixture of 2.79 Gm. (0.01 mole) of II, 50 ml. of dry benzene and 3 ml. of anhydrous pyridine there was added 3.42 Gm. (0.02 mole) of β-dichloroacetamidoethanol obtained above in small portions over a 30-min. period. The reaction mixture was stirred an additional 10 minutes. A heavy gum formed in the bottom of the reaction vessel. The supernatant liquid was decanted and the gum was washed with anhydrous ether. The gum was dissolved in 50 ml. of 95% ethanol and filtered. To the clear, yellow filtrate was added about 1–2 ml. of distilled water with shaking. The flask was allowed to stand overnight. The next day, the pale yellow crystals were removed on a Buchner funnel, dried, and recrystallized from ethanol, yielding 3.85 Gm. (70%) of product; m.p. 111–112°.

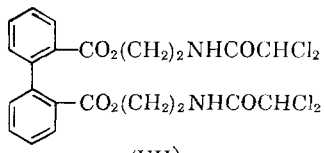
Anal.—Calcd. for $C_{22}H_{20}Cl_4N_2O_6$: N, 5.09; Cl, 25.82. Found: N, 5.08, 5.08; Cl, 25.76, 25.83.

N,N' - Bis(benzenesulfonyl) - *o,o'* - diphenoylurea, VIII.—In a 50-ml. Erlenmeyer flask was placed 1.4 Gm. (0.005 mole) of II and 2.0 Gm. (0.01 mole) of benzenesulfonylurea and the contents were thoroughly mixed. The flask was placed in an oil bath which was heated to and maintained at 130° for 2.5 hours. At this point, the bath temperature was raised to 160° and was kept between 160–170° for 6.5 hours. The flask was removed from the heating bath and allowed to come to room temperature. The crude product was recrystallized from absolute alcohol. A yield of 1.9 Gm. (64%) of product was obtained; m.p. 124–126°.

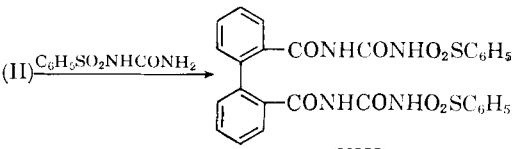
Anal.—Calcd. for $C_{28}H_{22}N_4O_8S_2$: N, 9.40; S, 10.74. Found: N, 9.28, 9.28; S, 10.57, 10.60.



(II)



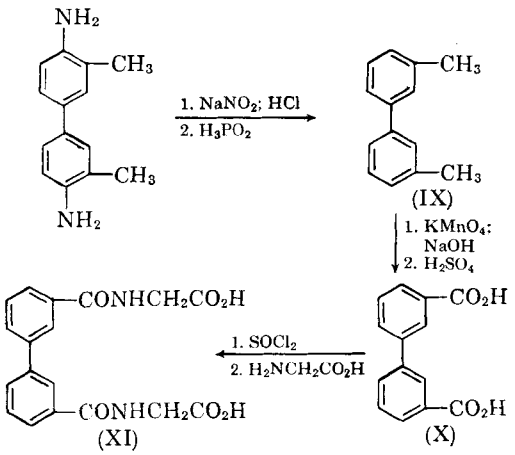
(VII)



(VIII)

***m,m'* - Bis(N - carboxymethyl)diphenamide, XI.**—*m,m'*-Bitolyl, IX, was prepared by the method described by Kornblum (11). The yield was 88% and the product distilled at 134–135° (6 mm.). A 4-Gm. quantity (0.022 mole) of IX, 15.8 Gm. (0.1 mole) of potassium permanganate, 2.0 Gm. (0.05 mole) of sodium hydroxide and 420 ml. of water were heated under reflux for a period of 12 hours. At this time, the solution was filtered while still hot and the filtrate carefully acidified by the slow addition of concentrated sulfuric acid. The white precipitate, *m,m'*-diphenic acid, X, after drying, weighed 3.2 Gm. (60%); m.p. 356–358°.

One gram (0.006 mole) of *m,m'*-diphenic acid, X, was refluxed with an excess (25 ml.) of thionyl chloride for 6 hours. The excess thionyl chloride was removed by distillation. The product was washed with successive portions of anhydrous benzene and dried. It weighed 1.2 Gm. One gram (0.004 mole) of the crude product obtained, 0.75



Gm. (0.01 mole) of glycine, and 1.0 Gm. (0.025 mole) of sodium hydroxide in 50 ml. of water were refluxed for 30 minutes. The mixture was cooled and the product precipitated by the slow addition of a 5% solution of hydrochloric acid and recrystallized from ethanol, yielding 1.1 Gm. of product (78.6%); m.p. 204° (decompn.).

Anal.—Calcd. for $C_{18}H_{16}N_2O_6$: N, 7.86. Found: N, 7.88, 7.89.

SUMMARY

Five new derivatives of diphenic acid have been prepared for pharmacological testing as possible antibacterial, antihyperglycemic, antispasmodic, and/or local anesthetic activity: *o,o'* - bis(2-nitro - 1,3 - dihydroxypropyl)biphenyl disodium salt; *o,o'* - bis(3 - benzoxy - 2 - benzamido - 1-hydroxypropyl)biphenyl; *o,o'* - bis(β - dichloroacetamidoethyl)diphenate; N,N' - bis - (benzenesulfonyl) - *o,o* - diphenoylurea, and *m,m'*-bis(N-carboxymethyl)diphenamide.

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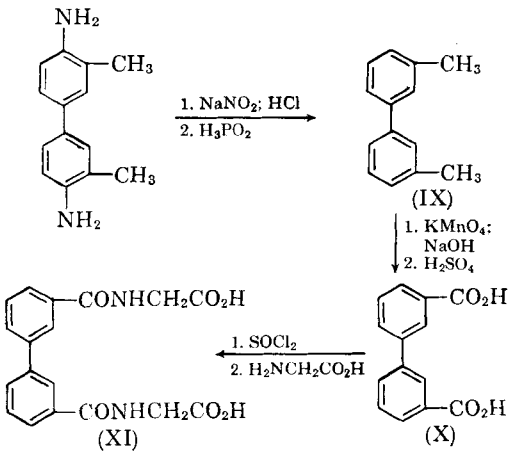
THE ANALYSIS of barbiturate salts in a variety of dosage forms by ion exchange and nonaqueous titration was recently reported (1). Application to the assay of elixirs proved unsuccessful. Ion exchange resins have been employed for the isolation of barbiturates (2) and a variety of nonaqueous techniques have been described for determining barbiturates in dosage forms. These have been reviewed in an earlier paper (1). The usual procedure in applying a nonaqueous titration procedure to the analysis of elixirs involves extraction of the barbiturate with an organic solvent prior to titration in a nonaqueous medium.

Potentiometric titration of phenobarbital elixir with silver nitrate was reported by Mattocks and Voshall (3) and later by Bodin (4). Cohen and Lordi (5) developed an amperometric and a potentiometric titration procedure with mercuric ion for analyzing the elixir. A general review of the literature dealing with the analysis of the barbituric acids is presented by Connors (6).

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This report describes a procedure in which the phenobarbital is removed from the elixir by

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passage through an anion exchange resin. The phenobarbital is eluted from the column with acetic acid in ethanol followed by titration in dimethylformamide with sodium methoxide in benzene-methanol. The assay is applied to other barbiturate elixirs.

EXPERIMENTAL

Preparation of Column.—The strongly basic anionic exchange resin Dowex 2-X8, 50-100 mesh, was used in this study. A suspension in water of about 10 Gm. of the resin was added to a chromatographic column, 1 cm. × 30 cm. The resin column was washed with 100 ml. of distilled water, 100 ml. of 5% sodium hydroxide, and 200 ml. of distilled water. The column was ready for use.

Assay Procedure for Pure Phenobarbital.—Approximately 100 mg. of pure phenobarbital, accurately weighed, was dissolved in 50 ml. of 50% ethanol. The solution was passed through the resin column. This was followed by the addition of 50 ml. of distilled water. The phenobarbital was eluted from the column with a solution of 50% acetic acid in 95% ethanol at a flow rate of 1 ml. per minute. The first 10 ml. of eluate contained no phenobarbital and was discarded. The next 50 ml. of eluate was collected and evaporated to dryness on a steam bath. Five milliliters of distilled water was added and the solution was again evaporated to dryness. This was repeated once. The residue was dissolved in 20 ml. of dimethylformamide and the solution was titrated with 0.1 *N* sodium methoxide in benzene-methanol prepared and standardized as described earlier (8). Titration was effected potentiometrically with a Fisher titrimeter equipped with a calomel and glass electrode system. The end point was determined by plotting millivolts *vs.* volume of titrant. The point of inflection was readily determined from the curve. A blank run was conducted with each series of three determinations. Recovery of phenobarbital was quantitative as indicated by data reported in Table I.

TABLE I.—ANALYSIS OF PHENOBARBITAL ELIXIR

Sample	Phenobarbital Theoretic or Labeled Amount, mg.	Proposed Method, % Recovery[b]	Official Method, % Recovery[c]
Pure phenobarbital	100.0	99.76 ± 0.09[d]	...
Elixir A	100.0[a]	99.12 ± 1.19	100.7
Elixir B	100.0	97.29 ± 0.77	99.5
Elixir C	100.0	96.15 ± 0.78	99.0
Elixir D	100.0	100.96 ± 0.29	104.7

[a] Mg. per 25 ml. [b] Average of at least 5 runs. [c] Average of duplicate runs. [d] Standard deviation.

Phenobarbital Elixir.—Exactly 25 ml. of phenobarbital elixir was transferred by pipet to a 150-ml. beaker. About 25 ml. of distilled water or 50% ethanol was added. This reduced the viscosity of the elixir and permitted a more rapid flow rate through the column. The solution was added to the column. When the last of the solution disappeared below the surface of the resin, distilled water was added to the column. The column was washed with 100-150 ml. of water. The phenobarbital was eluted from the column with 50% acetic acid in 95% ethanol. The eluate was treated as described previously for the pure phenobarbital sample. The data for the analysis of phenobarbital elixir are reported in Table I. Elixirs *A* and *B* represent different batches of phenobarbital elixir prepared in the manufacturing pharmacy of our pharmacy department. Elixirs *C* and *D* were commercial samples.

Other Barbiturate Elixirs.—The proposed assay procedure was applied to the analysis of elixirs containing secobarbital, pentobarbital, and amobarbital. Commonly used commercial varieties of the elixirs were employed in this study. These were treated in the same manner as described for phenobarbital elixir. The data are reported in Table II.

TABLE II.—ANALYSIS OF OTHER BARBITURATE ELIXIRS

Sample	Barbiturate Concn. Labeled Amount, mg.	% Recovery
Secobarbital elixir	110.0[a]	101.86 ± 0.49[c]
Amobarbital elixir	110.0	99.74 ± 0.69
Pentobarbital sodium elixir	91.3[b]	103.83 ± 1.07

[a] Mg. per 25 ml. [b] As pentobarbital. [c] Standard deviation.

DISCUSSION

The shortcomings of the official assay procedure for phenobarbital elixir have been noted here and have been indicated previously by other workers. Although a variety of methods and modifications have been proposed which appear to be superior to the official assay procedure the latter has remained essentially unchanged over the years.

In the technique proposed in the present study, a strongly basic anion exchange resin, quaternary base type, is used to remove the barbiturate from the other components of the elixir. This obviates the tedious and time consuming extraction process used in the official assay and in most of the proposed methods based on nonaqueous titration. The eluent, 50% acetic acid in ethanol, effectively removes the barbiturate from the column. Acetic acid, a stronger acid than the barbiturates, displaces the latter from the resin. Although the barbiturate may be quantitatively recovered from only 30 ml. of eluate, 50 ml. is collected in the assay to ensure complete extraction. Quantitative recoveries are reported for phenobarbital elixir in Table I and for other barbiturate elixirs in Table II. Comparison was made with the official assay for phenobarbital elixir. The data are shown in Table I. It should be noted that somewhat higher results were consistently obtained with the official procedure. This is undoubtedly due to the extraction of chloroform soluble components in addition to the phenobarbital. This was reported earlier by others (3, 7).

The interference by other components in the elixirs was studied. Nonionic ingredients such as sucrose, glycerol, and propylene glycol were readily washed

from the column with water. They appeared in the eluate. The amaranth coloring agent used in phenobarbital elixir did not pass through the column, but concentrated in the upper 0.5 cm. of the resin column. When the resin was to be regenerated, the upper centimeter of the column was removed and discarded. The column was then washed with distilled water, sodium hydroxide solution, and distilled water until the eluate showed pH 5 to 6. The column was ready for a second run. Caramel, the coloring agent used in certain barbiturate elixirs, appeared to be washed completely from the column with water. Barbiturate salts, as pentobarbital sodium in pentobarbital elixir, do not complicate the assay since only the anion is retained by the resin; the cation appears in the eluate. Synthetic sweetening agents as saccharin sodium and cyclamate calcium may be used in commercial barbiturate elixirs. A mixture containing 100 mg. of saccharin and 100 mg. of phenobarbital was titrated in dimethylformamide with sodium methoxide. Differentiation of the two acids was noted. This is shown in Fig. 1, curve *A*; the first inflection is due to saccharin and the second is attributed to phenobarbital. Recovery was quantitative for both components. A mixture of 100 mg. of saccharin sodium and 100 mg. of phenobarbital was dissolved in 50% ethanol and passed through the resin column. The column was eluted with acetic acid in ethanol as described for the barbiturate elixirs. Titration in dimethylformamide with sodium methoxide showed quantitative recovery of the barbiturate. See Fig. 1, curve *B*. Elution of the column with 1 *M* hydrochloric acid in 50% ethanol, evaporation of the solvent, and subsequent nonaqueous titration indicated quantitative recovery of the saccharin. Since acetic acid is a stronger acid than the barbiturates used in this study, but weaker than saccharin, the former and not the latter is displaced from the resin column. Similar results were obtained with cyclamate calcium. Hydrochloric acid, found in pentobarbital elixir, does not interfere since acetic acid will not displace it from the column. Salts, in general, will not interfere unless the anion represents an acid which is weaker than acetic

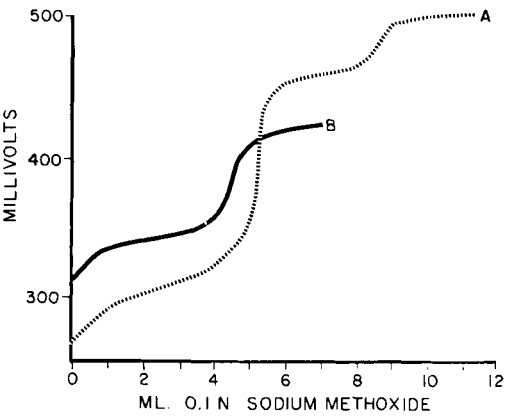


Fig. 1.—Curve *A*, titration of saccharin and phenobarbital mixture; curve *B*, titration of phenobarbital after passage of mixture through resin column.

acid. Even then, it may be possible to differentiate between it and the barbiturate by nonaqueous titration if the two differ significantly in acid strength. Methenamine and bases do not interfere.

The proposed assay procedure is simple, accurate, and less time consuming than the official procedure. It should have wide application. Although it has been used only for elixirs in this study, it should be applicable to other dosage forms and to certain combinations of barbiturates with other agents.

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Rheology of Thixotropic Montmorillonite Dispersions I

Changes on Aging of Plain and Polysorbate 80 Containing Dispersions

By GERHARD LEVY

The rheological characteristics of thixotropic montmorillonite dispersions are best defined on the basis of a concept of two different yield values, dynamic and static, in addition to plastic viscosity and thixotropic area. The values of these parameters were determined periodically for 10 weeks after preparation of the dispersions and were found to change at different rates. This shows that evaluation of a single rheological parameter as a function of time is not indicative of the overall rheological changes undergone by these systems. The feasibility of selective modification of certain rheological properties and aging characteristics is demonstrated by the rheograms obtained from montmorillonite dispersions to which polysorbate 80 had been added at the time of preparation.

SYSTEMS capable of isothermal gel to sol transformation upon shaking or shearing, and reversion to a gel upon rest, have considerable utility in pharmacy. Prominent among substances which can produce this property, known as thixotropy, upon dispersion in an appropriate liquid are the clays, particularly those of the montmorillonite group. The present study deals with a characterization of various rheological properties of a widely used member of the montmorillonite group, colloidal magnesium aluminum silicate (MAS), which is a refined product derived from saponite (1). Aqueous dispersions of this material exhibit complex time-dependent flow properties which are complicated further by certain irreversible changes upon aging. It has been found necessary to redefine the concept of yield value as applied to these systems, and to extend the interpretation of the obtained rheograms on the basis of the more recent rheological research results which apparently have not yet been taken into consideration by many pharmaceutical scientists. Of particular interest have been the quantitative and qualitative nature of the irreversible rheological changes of aqueous MAS dispersions upon aging, since it was hoped that subsequent studies in this series could be carried out with dispersions which have attained structural equilibrium. Since both reversible and irreversible changes of montmorillonite clays are related to the surface properties of these materials, it has been found desirable also to investigate the effect of a typical nonionic surface-active agent on the rheological and aging characteristics of the dispersions.

Received March 22, 1962, from the University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.
Accepted for publication May 31, 1962.
Presented to the Scientific Section, A.Ph.A., Las Vegas meeting, March 1962.
The author acknowledges the technical assistance of J. M. Rutowski.

EXPERIMENTAL

Preparation of Dispersions.—Three per cent w/w dispersions of colloidal magnesium aluminum silicate (Veegum)[1] were prepared in 500-Gm. portions by adding 15 Gm. of the clay to 485 ml. of distilled water, and mixing these vigorously for 3 minutes in an Arnold automatic mixer, model No. 15. The individual portions were combined while still hot and agitated with a Brookfield counter-rotating mixer in a 2-gal. beaker at a rheostat setting of 80 for 5 minutes, followed by 25 minutes of agitation at a rheostat setting of 50. The dispersion was then weighed and water lost by evaporation was replaced.

Water at room temperature was used in the preparation of dispersions by the "cold" process, while water heated to 80° was used in the "hot" process.

Polysorbate 80 was added after preparation of the dispersions by the "hot" process and incorporated by mild mixing to prevent foaming.

Storage.—The dispersions were placed in glass jars which were stored in an electric oven at a temperature of 30 ± 1°.

Instrumentation and Methodology.—The Epprecht Rheomat 15[2] was used to obtain the rheograms. This instrument is a rotational viscometer having 15 different shear rate settings for a given measuring system. Changes of shear rate are discontinuous and made manually, but do not require stopping the spindle. All measurements reported in this paper were made at 30°, using the "B" measuring system. The 15 shear rate settings were 3.110, 4.177, 5.496, 7.334, 9.674, 13.94, 18.72, 24.64, 32.87, 43.36, 62.94, 84.51, 111.2, 148.3, and 195.7 sec.$^{-1}$. Each experimental point in Figs. 1–3 represents an average value obtained from three rheograms of different samples of the dispersion. These samples were not returned to the stock dispersion, but discarded after use.

To obtain data for a rheogram, the viscometer cup was filled with an appropriate quantity of the dispersion and the spindle was immersed. Both were then attached to the instrument which was

[1] Manufactured by R. T. Vanderbilt Co., New York, N. Y.
[2] Made by Contraves AG, Zurich, Switzerland.

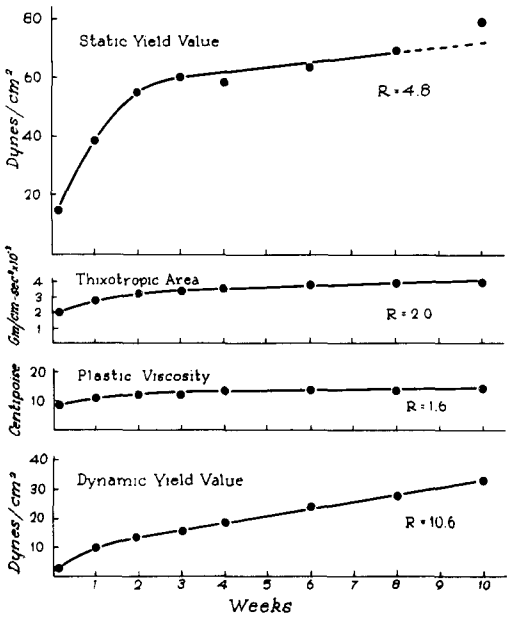


Fig. 1.—Changes in rheological properties of a 3% aqueous dispersion of MAS prepared by the "hot" process, during storage at 30° for 10 weeks.

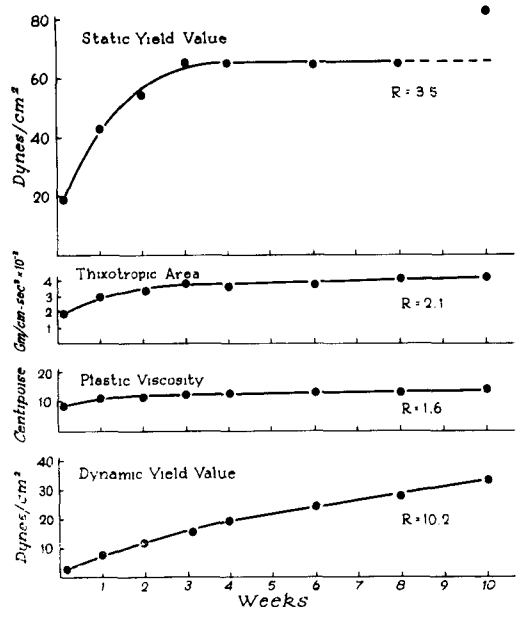


Fig. 2.—Changes in rheological properties of a 3% aqueous dispersion of MAS prepared by the "cold" process, during storage at 30° for 10 weeks.

positioned so that the cup was immersed in a constant temperature water bath set at 30 ± 0.1°. The dispersion was sheared at a rate of 195.7 sec.$^{-1}$ for 5 minutes and then permitted to remain undisturbed for 30 minutes. During this time, the cup was covered with a plexiglas disk to prevent significant evaporation of water from the surface of the dispersion. At the end of the 30-minute period, the viscometer spindle was set in motion at the lowest shear rate. Characteristically, the shear stress indicator exhibited a large excursion as the spindle began to rotate. This maximum torque was noted. Another reading was made 30 seconds after the spindle had been set in motion. The shear rate was then increased to the next setting, and shear stress measured after 30 seconds. Shear rates were increased at 30-sec. intervals until the highest shear rate was reached, then decreased at the same rate to the lowest shear rate. The shear stress data obtained at the various shear rates were used to draw the rheograms.

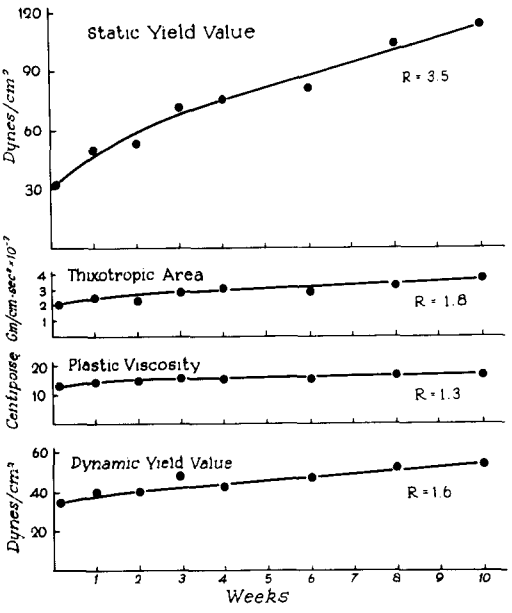


Fig. 3.—Changes in rheological properties of a 3% aqueous dispersion of MAS prepared by the "hot" process and containing 0.1% polysorbate 80, during storage at 30° for 10 weeks.

Yield value corrections were made according to Epprecht (2), using a K_f factor of 0.80, which is based on an R^2/r^2 value of 1.58. Thixotropic areas were determined by means of a compensating polar planimeter from a graph where the shear rate axis represented 7.75 sec.$^{-1}$ per cm. and the shear stress axis 24.9 dynes/cm.^{2} per cm. Accordingly, a thixotropic area of 1 cm.^{2} was equal to 193 Gm./cm. sec.^{3}. The thixotropic area was taken as the area enclosed by the ascending and descending shear rate *vs.* shear stress curves, and the horizontal line connecting the experimental points of both curves at a shear rate of 3.110 sec.$^{-1}$, as shown in Fig. 4.

RESULTS AND DISCUSSION

Flow curves of thixotropic systems are defined usually on the basis of plastic viscosity, thixotropic area, or index, and the value of the shear-stress intercept of the extrapolated linear portion of the down curve at zero shear rate. The latter value is referred to as the yield point or yield value. The rheograms of the MAS dispersions yielded up curves having characteristic shear stress maxima at the

beginning of measurement, followed by shear stress minima at a relatively low shear rate (Fig. 4). The shear stress maxima have been observed only in re-

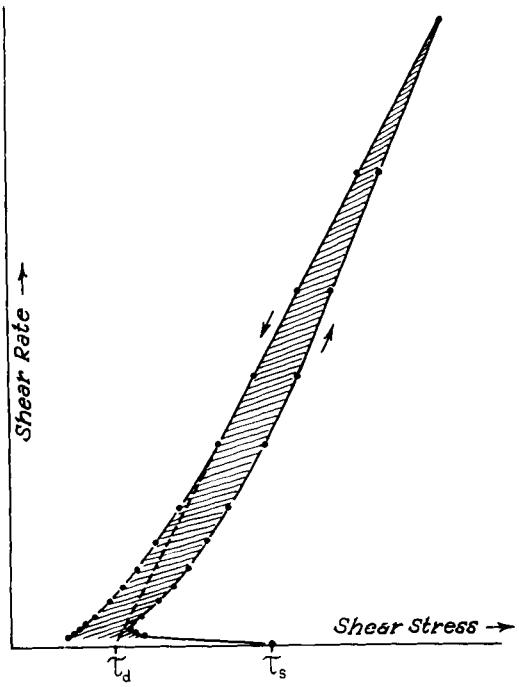


Fig. 4.—Typical rheogram of an aqueous dispersion of MAS. τ_d, dynamic yield value; τ_s, static yield value. Shaded section represents the thixotropic area.

cent years when continuous recording viscometers and more refined and sensitive multiple-point instruments have come into use. However, there is already evidence that these maxima reflect a rheological property of direct pharmaceutical pertinence; for example, it has been found that the persistence of penicillin levels in the blood after administration of procaine penicillin depot preparations is directly related to the value of the initial shear stress maximum of these suspensions (3). Thus, it is desirable to consider the initial shear rate maximum as one of the several rheologic properties which must be measured in order to describe a thixotropic system adequately. This necessitates a redefinition of the concept of yield value as well as a consideration of the significance of the shear stress minimum on the up curve of the rheogram.

Conventional or Dynamic Yield Value.—The shear-stress intercept of the linear portion of the down curve of a rheogram at zero shear rate, referred to conventionally as the yield value, is usually defined as the minimum tangential shearing force necessary to cause flow (4). Experimentally, however, it can be shown that the shearing force necessary to initiate flow of thixotropic montmorillonite dispersions is considerably higher than the conventional yield value. This is reflected by the initial shear stress maximum characteristic of such dispersions. Furthermore, if the conventional yield value really has the meaning assigned to it by the above definition, it should be independent of the maximum shear rate to which the system is exposed in the course of measurement. Such is not the case since the yield value frequently increases upon increasing the top shear rate used in the determination of the flow curve (4, 5). It is well appreciated that the need for some minimum shear stress to initiate the flow of thixotropic systems is predicated upon the presence of a continuous structure whose interparticle bonds must be broken in the process of inducing flow. Thus it should be clear that any measurement based solely on a thixotropic system in flow cannot provide information concerning the shear stress necessary to cause this flow. Upon examination of a typical rheogram, the real significance of the conventional yield value becomes quite clear; it is the energy-input necessary to maintain a constant ratio of shear rate to shear stress (that is, total shear stress minus the yield stress referred to) in a system whose structure has been destroyed, totally or in part, by previous shear (in the course of determining the up curve of the rheogram). Expressed in a different way, the conventional yield value reflects the energy input required to maintain the degree of structural breakdown which exists at the maximum shear rate, just before shear rate is reduced for the determination of the down curve. This is reasonably analogous to the concept of a coefficient of dynamic friction as opposed to the coefficient of static friction of solids. Accordingly, it is proposed that the conventional or Bingham yield value be referred to as the dynamic yield value, unless it can be shown experimentally that this value does not differ from the yield value necessary to cause flow (which would be the case in plastic systems). Apart from the purely mathematical usefulness of quantitating the dynamic yield value, it should also be indicative of the degree to which the internal structure of a system has been destroyed by a given shear stress and duration of shear. At some point, a further increase in shear stress and/or duration should cause no further increase in dynamic yield value; namely, when all the weak secondary bonds between particles have been broken and the dispersed units consist solely of primary particles.

Static Yield Value.—This term evolves as a logical consequence of accepting the concept of a dynamic yield value. It may be defined as the minimum tangential shearing force necessary to initiate flow. Unfortunately, this value is not independent of time. Houwink has pointed out the necessity of including a specification about the rate of increasing shear stress when defining yield value (6).[3] McVean and Mattocks (7) could demonstrate that the static yield value is a function of rate of strain, since it is evident that their rising sphere rheometer measures static rather than dynamic yield value at the "flow" point. The time dependency stems from the viscoelastic and relaxation behavior of most gels. In stating that the force required to break down structure and cause flow of thixotropic suspensions is time-dependent and must be represented by a curve with time as one of the parameters, Dintenfass points out that it is not a case of no flow occurring below a given shear stress value, but rather that the time taken for measurable flow to take place would be abnormally long (8). In addition, the time effect

[3] Within the context of Houwink's discussion it is clear that he was referring to the "static" yield value.

applies also to the rest period of the thixotropic system. In the act of charging the viscometer, structure is destroyed and requires time to rebuild, as indicated by data reported in the following paper (9). Moreover, there are indications that the structural recovery rate is affected by the diameter of the annulus of the viscometer (9). The static yield value is, therefore, far from an absolute value and is related not only to the previous history of the measured material, but also to measurement conditions and instrument characteristics. While the concept of a dynamic and a static yield value has, to the best of our knowledge, not previously been applied experimentally within the strict definition of these terms as presently proposed, it is of interest to note that the idea is not new. A review of the literature has shown that Green and Weltmann suggested these terms in 1946 but evidently did not follow upon their suggestion with experimental data, possibly due to lack of appropriate instrumentation (5).

Shear Stress Minimum.—Prominent in the beginning portion of the up curve of the thixotropic rheogram shown in Fig. 4 is a shear stress minimum, which may occur at shear rates significantly higher than zero. This phenomenon has been explained recently by Enneking (10). He found that the shear stress minimum occurred at higher shear rates when the annular width of the viscometer was increased. As the spindle begins to move (*i.e.*, the point at which static yield value is determined), the measured material flows only at the spindle surface and not throughout the annular space. Upon increasing the spindle velocity, further breakdown of structure occurs and the effective annular distance increases. Since shear rate is inversely proportional to the distance between annular boundaries, the actual shear rate to which the thixotropic material is subjected is decreased, despite the higher speed of rotation of the spindle. Naturally, the shear stress will be less at this lower shear rate. This process continues until flow occurs throughout the space between cup and spindle. At that point, the shear stress minimum is reached and any further increase in spindle r.p.m. reflects a true increase in shear rate and, therefore, requires higher shear stresses. The narrower the annulus, the lower the shear rate at which the stress minimum will occur. Thus, this factor is primarily an instrumental characteristic rather than a property of the material, although the rate of thixotropic breakdown is a contributing factor under the usual nonequilibrium experimental conditions. The experimental points on the up curve of the rheogram between the static yield value and the shear stress minimum have, therefore, no absolute physical meaning since they are plotted on the basis of apparent, but not actual, shear rates.

Rheological Changes of Montmorillonite Dispersions on Aging.—Figure 1 shows the changes in static yield value, thixotropic area, plastic viscosity, and dynamic yield value of a 3% MAS dispersion prepared by the "hot" method during a period of 10 weeks after preparation. Static yield value increased quite rapidly during the first 3 weeks and then leveled off. The R value in the graph is the ratio of the 10-wk. value to the 24-hr. value.[4] For this calculation, the 10-wk. value indicated by the extrapolated curve was used, since the actual experimental points in Figs. 1 and 2 are unusually high and appear to be artifacts. Thixotropic area also increased most rapidly during the first 3 weeks and very slightly from then on. Over the 10-wk. period, the thixotropic area increased only twofold, as opposed to an almost fivefold increase in static yield value. The plastic viscosity exhibited an even smaller change, and essentially reached equilibrium after 4 weeks when it had increased by 60% over the 24-hr. value. This rather small increase is readily understandable if it is assumed that the thixotropic suspension, at the time of measurement of the down curve, consists of individual particles (not necessarily primary particles) suspended in a Newtonian liquid (water). This assumption is reasonable since interparticle bonds have been broken by previous shear and are prevented from reforming as long as the down curve remains linear. As a first approximation, the viscosity of such a liquid should follow the Einstein equation

$$\eta = \eta_0 (1 + 2.5\phi)$$

where η is the viscosity of the suspension, η_0 the viscosity of the medium, and ϕ the volume fraction of the dispersed phase. The latter value would increase somewhat in an aging clay dispersion due to lattice expansion, the degree of this expansion being a function (among others) of the moisture content of the solid clay just prior to use.

Dynamic yield value increased over tenfold during 10 weeks. This is remarkable, especially since the dispersion was agitated quite intensively in the course of preparation. The increase in dynamic yield value signifies that a greater energy input is necessary to prevent structure reformation as the dispersion ages. This may be due to the ultimate dispersion into primary particles of what were originally face-to-face aggregates of numerous clay particles. These primary particles should be capable of individual participation in the formation of the typical scaffolding structure of clay dispersions at rest.

The rheological changes during aging of MAS dispersions prepared by the "cold" method did not differ significantly from those of the dispersions prepared by the "hot" process (Fig. 2). This result was somewhat surprising since the use of heat is generally recommended for the preparation of montmorillonite dispersions. Reference to the literature indicates, however, that another worker also observed only small temperature effects with MAS (11). It may be that the temperature effect is greater when the dispersions are subjected to lower agitation intensities and shorter agitation periods during preparation.

Effect of Polysorbate 80.—The addition of 0.1% polysorbate 80, immediately after preparation, to 3% MAS dispersions prepared by the "hot" method results in a profound modification of flow and aging characteristics (Fig. 3). The static yield value at 24 hours is about 50% higher than in the absence of surfactant. It increases at practically constant rate throughout the 10-wk. period. The thixotropic area is somewhat smaller in the polysorbate 80-containing dispersions. This could be due either to a smaller degree of thixotropy or to a more rapid

[4] This R should not be confused with the coefficient of thixotropic recovery, also designated as R, in the following paper (9)

structural recovery. Subsequent studies described in the following paper (9) indicate that a more rapid structural recovery rate is probably the reason for the smaller thixotropic area. The plastic viscosity is slightly higher in the presence of polysorbate 80. Since polar but otherwise neutral molecules, such as the glycols, are known to be adsorbed between the silicate laminae of montmorillonite clays (12, 13), it is probable that the higher plastic viscosity of polysorbate 80-containing dispersions is due to an increased volume of the dispersed phase caused by lattice expansion upon adsorption of the surfactant. The most significant effect of surfactant was, however, a tenfold increase in dynamic yield value at 24 hours. The subsequent increase upon aging was only 60%, compared with a tenfold increase in the dispersions without surfactant. The presence of polysorbate 80 thus affects, primarily, the dynamic and static yield values, and only slightly the thixotropic area and plastic viscosity of aqueous MAS dispersions. This demonstrates the possibility of a selective modification of certain rheological properties by means of additives capable of modifying the surface properties of montmorillonites. Interpretation of the mechanism of the polysorbate 80 effect is complicated by the fact that the montmorillonite was neither dialyzed nor homoionic. It is known that neutral but polar molecules adsorb on the surface of montmorillonite, orient parallel to that surface, and are capable of displacing hydrogen-bonded water (13). It was found in the course of studies of the adsorption of nonionic aliphatic molecules from aqueous solutions on montmorillonite, that mixtures with high molar adsorption tend to form gels while mixtures with low molar adsorption settle out on standing (14). Of interest, also, are the observations by Samyn that small concentrations of polysorbate 40 increase the apparent viscosity of MAS dispersions but that higher surfactant concentrations reverse this increase (15). It is possible that the surfactant effect is due to partial displacement of inorganic cations from the surface of the clay, although such displacement need not necessarily occur when organic molecules are adsorbed (16). It has been suggested also that surfactant micelles can serve as a locus for the attachment of individual colloidal particles (17). The surfactant may, therefore, have a bridging effect. The high initial yield values and smaller subsequent changes (as compared with those of suspensions without the surfactant) indicate that the lowering of interfacial tension by the surfactant could have increased the rate of dissociation of face-to-face aggregates of the clay. This would make a larger number of particles available for face-to-edge or edge-to-edge association which leads to the scaffolding structure responsible for high yield values. Experiments are currently under way to elucidate the mechanism or mechanisms of the surfactant effect.

CONCLUSIONS

The experimental data show that aqueous dispersions of a representative montmorillonite clay undergo large rheological changes on aging. It has not been possible under our experimental conditions to attain structural equilibrium during 10 weeks of aging at 30°. Other studies in this laboratory indicate that even considerably longer aging periods are inadequate for equilibration of rheological properties of montmorillonite dispersions. The changes in rheological properties of montmorillonite dispersions on aging or due to additives can be characterized adequately only if all the various rheological parameters are determined individually, since they change to different extents. Comparative studies on montmorillonite dispersions must be standardized rigidly with respect to time in order to exclude the aging period as one of the variables. The effect of polysorbate 80 on the rheological and aging properties of MAS dispersions illustrates the feasibility of a relatively selective modification of these properties by means of additives for the purpose of obtaining optimum flow and aging characteristics in a pharmaceutical system.

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Rheology of Thixotropic Montmorillonite Dispersions II

Kinetics of Structural Recovery

By GERHARD LEVY

Methodology has been developed to study the kinetics of the structural recovery (build-up) of thixotropic montmorillonite dispersions. The recovery process can be described by means of a coefficient of thixotropic recovery with time, R. Thus: $R = (T_2 - T_1)/(\log t_2 - \log t_1)$, where T_2 and T_1 are the torques necessary to overcome the resistance encountered by a special spindle which is simultaneously rotated and lowered into the dispersion at times t_2 and t_1, respectively, after breakdown of structure by agitation. The effects of aging, concentration, method of preparation (hot process, cold process, ultrasonic treatment), and addition of polysorbate 80, zinc oxide, or glycerin upon structural strength (static yield value) and coefficient of thixotropic recovery have been determined.

THE USEFULNESS of thixotropic systems in pharmacy is based upon their ready conversion from a rigid gel to a fluid sol upon shaking or shearing, and their reversion to the gel state when shaking or shearing is discontinued. The sol to gel transformation or structural recovery is not instantaneous since the particles in random motion are required to approach one another from the proper direction and to overcome an energy barrier before interparticle association can occur. The structural recovery rate of thixotropic systems is probably the most important variable with respect to their pharmaceutical usefulness. For example, if recovery occurs too quickly, thixotropic suspensions will not remain fluid for a sufficient time after agitation to permit pouring. On the other hand, if the time required for structural recovery is too long, the suspended particles will descend to the bottom of the container instead of becoming immobilized within a structural network. This would result in a "stabilized sediment" rather than a well-dispersed, physically stable suspension.

The lack of investigation by pharmaceutical scientists of this important aspect of the rheology of thixotropic systems may be due partly to the long-prevailing misconception regarding the significance of the conventional yield value (1). Usually defined as the minimum tangential force necessary to initiate flow, it has been determined in dynamic systems rather than in static or nonflowing systems. The conventional definition of the term as well as the method used to measure it imply that the yield value remains the same regardless of the amount of shear to which the thixotropic system is subjected. Thus, there would be no reason to assume that yield value will decrease upon shearing and increase during rest, and any logical basis for studying structural recovery rates would be nonexistent.

Redefinition of the conventional yield value as dynamic yield value (which is considered to be indicative of the energy input required to prevent structure reformation), and postulation of a static yield value as the tangential force necessary to break structure to the point of initiating flow (1), provide a logical foundation for a study of thixotropic recovery rates.

Determinations of thixotropic recovery rates require repeated measurements of static yield value in a resting system as a function of resting time. In the early studies of thixotropic phenomena, the so-called "inverted tube method" was most commonly used to determine gelation or setting time (2), and the reciprocal of this value was taken as the rate of gelation (3).

The setting time of a thixotropic system was defined as the time between liquefaction, by mechanical action, and its resetting upon standing at rest. A sample, kept in a test tube, was considered to have set if it did not flow within a minute or so upon turning the tube upside down (4). Apart from various technical difficulties such as the need for a standardized inversion procedure (5), it was soon realized that the setting time is just an arbitrary point on the gel-strength *vs.* time curve, and that it does not reflect any sudden change in slope or other unique characteristic of such a curve (3, 6). It was also found that the diameter

Received March 22, 1962, from the University of Buffalo School of Pharmacy, Buffalo 14, N. Y.
Accepted for publication May 31, 1962.
Presented to the Scientific Section, A.Ph.A., Las Vegas meeting, March 1962.
The author acknowledges the technical assistance of J. M. Rutowski.

of the tube had a very large effect on setting time. For example, a bentonite dispersion with a setting time of 30 seconds in a tube of 13 mm. diameter, exhibited a setting time of over 3 hours in a tube having a diameter of 40 mm. (2). The container wall apparently has an organizing effect upon the clay particles, as has been suggested by Houwink (7). This wall effect also limits the usefulness of the standard rotational viscometers for the determination of thixotropic recovery rates, although several investigators have used such instruments to determine what is here defined as static yield value as a function of resting time (8, 9). Evidently these workers did not consider the effect of annular diameter upon thixotropic recovery rate or they felt that this factor was not important relative to the objective of their studies. For pharmaceutical purposes, however, it is desirable to obtain a realistic indication of recovery rates of the material under study when contained in vessels of relatively large diameter. Data to be presented here indicate that recovery rates under these conditions differ by several orders of magnitude from those in which the thixotropic material was situated within a narrow annular space.

A new methodology to determine thixotropic recovery rates, which permits measurements in containers of relatively large diameter where recovery rates become essentially independent of vessel diameter, has been developed for this study. It has also been possible to follow the change in static yield values over long periods of time since the thixotropic material could be stored in regular containers until and during the time of measurement. Apart from a description of the method, this communication deals with a mathematical characterization of the thixotropic recovery process, and a survey of the effect of different preparatory procedures and additives on the static yield value and thixotropic recovery rate of aqueous dispersions of magnesium aluminum silicate (MAS), a representative clay of the montmorillonite group.

EXPERIMENTAL

Preparation of Dispersions.—Dispersions, 3% w/w, of colloidal magnesium aluminum silicate (Veegum)[1] were prepared by adding 15 Gm. of the clay to 485 ml. of distilled water and mixing these vigorously for 3 minutes in an Arnold automatic mixer, model No. 15. The individual portions were combined and agitated with a Brookfield counter-rotating mixer in a 2-gal. beaker at a rheostat setting of 80 for 5 minutes, followed by 25 minutes of agitation at a rheostat setting of 50. The dispersion then was weighed and water lost by evaporation was replaced.

Dispersions of 4, 3.5, and 2.5% concentration were prepared in the same manner as the 3.0% dispersion, except for the necessary changes in amounts of the clay and water.

For dispersions prepared by the "hot" process, the MAS was dispersed in water previously heated to 80°, while water at room temperature was used in the "cold" process. For ultrasonic treatment, 200-ml. portions of dispersion were cooled to 15° and insonated in a General Electric ultrasonic generator, model G 3, at a frequency of 750 kc./sec. for 5 minutes. The temperature of the dispersions did not rise above 25° during insonation. The individual portions of dispersion were subsequently combined. Dispersions containing zinc oxide were prepared in the usual manner, but the zinc oxide was incorporated just 3 days before rheologic measurement, *i.e.*, 9.5 weeks after preparation of the plain dispersion. The zinc oxide was passed through a 60-mesh sieve prior to use.

Polysorbate 80 was added either to the completed dispersion immediately after preparation or it was dissolved in the water prior to addition of the clay.

Glycerin-containing dispersions were prepared by dispersing MAS in water in the usual manner, and adding glycerin just 3 days prior to rheologic measurement, *i.e.*, after 9.5 weeks of aging the plain dispersions.

All dispersions were stored in 8-oz. glass jars (5.5 cm. diameter, 13 cm. height) at room temperature (24 ± 2°). With the exception of the aging experiment (Fig. 3), the dispersions were aged for 10 weeks prior to rheologic evaluation.

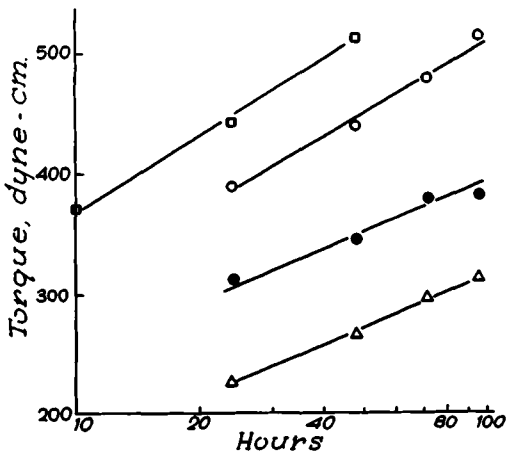


Fig. 1.—Effect of age on static yield value and thixotropic recovery rate of 3.0% MAS dispersions. Δ, 2 Weeks; □, 6 weeks; ○, 11 weeks; ■, 17 weeks.

Instrumentation and Methodology.—The Brookfield Synchro Lectric viscometer, model LVT, mounted on a Brookfield Helipath stand, was used. The spring tension (torque) of the instrument is 673 dyne-cm. at full scale, with a linear relation between

[1] Manufactured by R. T. Vanderbilt Co., New York, N. Y.

scale readings. A No. 5 Helipath spindle, consisting of a 11.4-cm. long stem having a diameter of 0.16 cm. with a 3.6-cm. long, 0.07-cm. diameter crossbar, 0.6 cm. from the end, was used for the measurements. This spindle was rotated at 6 r.p.m. while the platform of the Helipath stand descended at a rate of 2.2 cm. per min. Consequently, the crossbar of the spindle passed through the system being measured in a helical path, continuously contacting a different and previously undisturbed region.

A jar containing 200 ml. of the dispersion was opened carefully in a manner which avoided jarring, and placed on a micro Lab-Jack beneath the viscometer. The instrument was lowered manually until the crossbar of the spindle just touched the dispersion. The Lab-Jack was then raised 1 cm., so that the crossbar was immersed in the dispersion to that distance. Both viscometer and Helipath stand were then switched on, and torque readings were taken at every revolution of the spindle for a total of 16 revolutions. At no time was the spindle stopped or the torque indicator clutch engaged during the period of measurement. The 4th to the 13th readings were averaged and are reported here. Thus, the data represent the structural strength found in the body of the dispersion rather than that found close to the surface or bottom.

To determine structural recovery rate, jars containing the dispersion were placed on a reciprocating shaker with a shaking excursion of 3.8 cm., and shaken at 280 oscillations per min. for exactly 1 minute. The jars then were stored and left undisturbed until time of measurement. Five jars were used at each measurement period, and their average individual readings were averaged to yield the experimental values for the torque *vs.* time plots reported here.

RESULTS AND DISCUSSION

The method used for the determination of structural strength satisfies the requirement for measurement of undisturbed structure, since the crossbar of the inverted T spindle continuously encounters a new region as the spindle descends in the dispersion. The structural resistance met by the spindle is indicated continuously by the instrument pointer in terms of scale units, which can be changed to conventional torque units. The instrumental design does not, however, permit this value to be expressed in terms of dynes/cm.^{2}. Thus, the static yield value determined by the instrument cannot be expressed in conventional units, but this presents no undue disadvantage with respect to the present studies. Since the dispersion can be measured in the container in which it is stored, it is possible to utilize a large number of samples at all time intervals without inconvenience. The reproducibility of the method is high, as indicated by the data shown in Table I, which were selected at random from the total tabulation of experimental results. It is desirable that the rate of application of force by the spindle be high compared with the relaxation time of the system. Reference to the relaxation time of a MAS suspension reported by McVean and Mattocks (10) indicates that this is the case. While it usually is desirable to carry out rate studies at constant temperature, the present studies were made at room temperature since storage of the jars in an oven or water bath would subject the dispersions to vibration and motion. It is known that oscillations and similar slight motion can increase the recovery rate of bentonite dispersions as much as several hundredfold (5), so that it is important not to expose the dispersions to such effects. The usual ambient temperature fluctuations (±2°) did not have a significant effect on the recovery rates of MAS dispersions.

TABLE I.—STATIC YIELD VALUE[a] OF A 3% MAS DISPERSION[b] AT 4 HOURS

Jar No.	Average	Range
1	28.64	28.6–28.7
2	28.40	All readings identical
3	28.79	28.6–29.0
4	28.90	All readings identical
5	29.84	29.8–29.9
Overall	28.9	28.6–29.9

[a] Expressed in instrument scale units. [b] Ten weeks old, prepared by the "hot" method.

Kinetics of Structural Recovery.—It was found that a linear relationship exists between static yield value of MAS dispersions and the logarithm of resting time (Fig. 2). This linear relationship can be expressed as

$$T = \frac{T_2 - T_1}{\log t_2 - \log t_1} \log t + \text{constant} \quad \text{(Eq. 1)}$$

where T is the torque on the spindle at time t, T_1 the torque at time t_1, and T_2 the torque at time t_2. The structural recovery rate, therefore, is reflected by a coefficient of thixotropic recovery with time, R, which can be defined as

$$R = \frac{T_2 - T_1}{\log t_2 - \log t_1} \quad \text{(Eq. 2)}$$

and is the slope of the line which correlates torque and logarithm of resting time. Differentiation of Eq. 1 yields

$$R' = \frac{dT}{dt} \cdot t \quad \text{(Eq. 3)}$$

where

$$R' = 0.434\,R \quad \text{(Eq. 4)}$$

From Eq. 3 it is evident that the rate of change of torque is inversely proportional to the time and that R' is the proportionality constant.

The definition of R in the above equations is identical with that of Chong and co-workers for a coefficient of increased resistance to shear with time, as applied to the increase in yield value with shearing time of antithixotropic systems (11). Thus it is possible to express the rate of structure formation in thixotropic systems at rest and in antithixotropic systems during shear by means of the same coefficient R. This lends added justification to the term "antithixotropy" as applied by Chong, *et al.*

It would seem reasonable to assume that the structural recovery process ceases after a certain maximum static yield value is attained. The maximum was not reached in the present studies during periods considerably longer than 100 hours. Measurements over even longer periods of time are com-

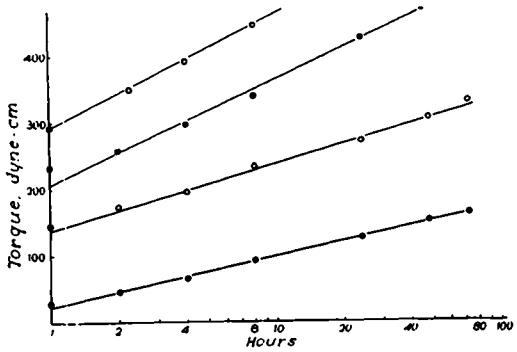


Fig. 2.—Structural recovery of aqueous dispersions of MAS. ●, 2.5%; ○, 3.0%; ■, 3.5%; □, 4.0% w/w.

plicated by the superimposed irreversible rheological changes due to aging, as described in the previous paper (1), which begin to overshadow and distort the now very small changes due to structural recovery. It has also been found that the initial stage of the thixotropic recovery process does not follow the kinetic scheme described. Reference to Figs. 2 and 3 will show that 1-hr. values were occasionally higher than theoretically indicated. Ten-minute values, not reported here, were always much higher than indicated by extrapolation of the straight line. This suggests that structural buildup occurs in two consecutive processes, analogous in part to von Smoluchowski's theory of fast and slow flocculation, for which assumption considerable evidence is available. Hauser and Reed have observed the structural build-up of bentonite dispersions with the ultramicroscope and have found that individual particles first form clusters which subsequently join together to form a continuous network throughout the dispersion (5). This two-step sequence has been observed also in other types of thixotropic dispersions. For example, Voet has found that structure formation in a dispersion of channel black in mineral oil occurred by association (formation of aggregates) followed by reticulation (interlinking of aggregates into a continuous network) (12). The data of Braune and Richter (8) also indicate that the initial rate of structural recovery of bentonite dispersions is higher than the rate in the subsequent

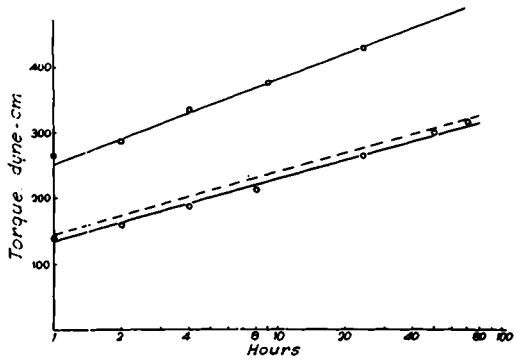


Fig. 3.—Effect of glycerin on static yield value and thixotropic recovery rate of 3% MAS dispersions. – – –, No glycerin; ○, 10% glycerin; □, 30% glycerin.

period. While the present studies deal with the kinetic aspects of structural recovery of MAS dispersions after the first hour of rest, the kinetics of the initial recovery process are currently under investigation and will be dealt with in a subsequent communication.

Effect of Concentration on Structural Recovery Rate.—Figure 4 shows that the logarithm of the coefficient of thixotropic recovery (R) is related linearly to the logarithm of MAS concentration. This relationship takes the form

$$\log R = a \log c + \log K \qquad \text{(Eq. 5)}$$

where c is the concentration of MAS, and a and K are constants. From the slope of the line fitted to the experimental data by the method of least squares, $a = 1.9$. It appears therefore, that

$$R = Kc^2 \qquad \text{(Eq. 6)}$$

since the experimental line deviates only slightly from a slope of 2.

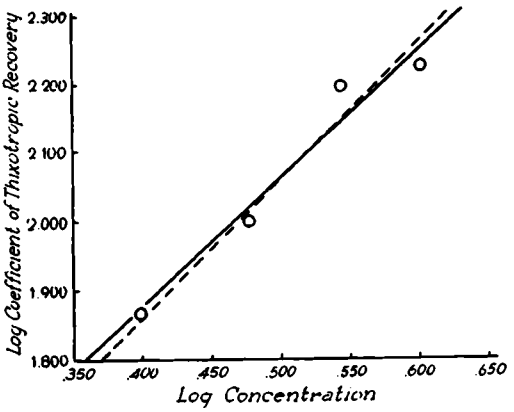


Fig. 4.—Effect of concentration on the coefficient of thixotropic recovery of aqueous dispersions of MAS. ———, Fitted line; - - - -, line with slope 2.

Effect of Aging.—Figure 1 shows the effect of aging on the recovery rate of 3% MAS dispersions prepared by the "hot" method. Both the recovery rate as well as the static yield value at any time during the measurement period increase as the dispersions age. Structural equilibrium had not been reached even after 17 weeks. The magnitude of the rheological changes on aging are, of course, related to the method of preparation of the MAS dispersions, particularly to the duration and intensity of shear to which the dispersions are exposed. The data presented in Fig. 1 were obtained from dispersions prepared from a different lot of MAS than was used in all other experiments reported here. Consequently, the line representing the structural recovery rate of a 10-wk. old dispersion shown in Fig. 2 does not "fit" into the plot and is closer to the 6-wk. line than to the 11-wk. line. This apparent discrepancy is not unusual since it is known that great lot-to-lot variations occur even in a relatively well-defined clay such as the type of MAS used in this study. The data of Samyn, for example, show a more than twofold difference in apparent viscosity between different lots of MAS hydrated for an equal length of time (13).

Method of Preparation.—No attempt has been made to study the effect of numerous manufacturing variables on MAS dispersions, since these have been described by Wood and co-workers (14) and by Simon and co-workers (15). In this study the structural recovery of MAS dispersions prepared by the "hot" method was compared with the recovery of MAS dispersions prepared by the "cold" method, in order to verify the surprisingly small effect of temperature noted in the first part of this investigation (1). The magnitude of the temperature effect again was found to be small (Fig. 5), which is in agreement with our previous results and also with the data reported by Vogt (16). The effect of ultrasonic waves on montmorillonite dispersions had not been studied by the authors cited previously (14, 15) and was, therefore, investigated. Cavitational heating of the dispersions was minimized by precooling and limiting the insonation period to 5 minutes in order to separate the possible temperature effect from the effect due to ultrasonic waves. The results show that ultrasonic treatment can increase the structural strength of MAS, probably due to greater dissociation of face-to-face particle aggregates (Fig. 5). Since the conditions of insonation were chosen arbitrarily, the results should be interpreted only in a qualitative manner.

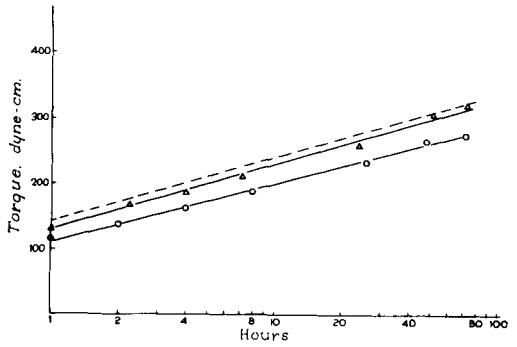


Fig. 5.—Effect of method of preparation on static yield value and thixotropic recovery rate of 3.0% MAS dispersions. O, "Cold" process; Δ, "cold" process and ultrasonic treatment; - - -, "hot" process.

Effect of Additives.—The effect of glycerin on the structural recovery rate and static yield value of MAS dispersions is shown in Fig. 3. In order not to superimpose a possible aging effect, glycerin was added to the MAS dispersions only 3 days prior to measurement, *i.e.*, after 9.5 weeks of aging. Glycerin had no significant effect at a concentration of 10%, but in the presence of 30% glycerin, both recovery rate and static yield value were considerably increased. Since the viscosity of a 30% aqueous glycerin solution is about 2.5 times higher than the viscosity of pure water, it was thought that the structural recovery rate would be lowered by glycerin due to the decreased mobility of dispersed MAS particles in a medium of higher viscosity. Such was not the case and the data suggest an increase in interparticle forces which may overshadow the viscosity effect. It is known that glycerin adsorbs on montmorillonites even from dilute aqueous solutions (17), and it has been suggested recently that the glycerin-bentonite structure differs from the structure of bentonite in water (18). Unfortunately, details of the latter study are not yet available and cannot be considered at this time.

It was of interest to study the effect of polysorbate 80 on MAS dispersions by the present method in view of the profound effect of this surfactant on the nature of the classical rheogram as noted in the first part of this investigation (1). Figure 6 shows the increased recovery rate and static yield value of MAS dispersions upon addition of 0.1% polysorbate 80. The increase in static yield value reflected by increased torques on the inverted T spindle parallels the increase in static yield value as determined with the standard rotational viscometer (1). The higher recovery rate of the dispersions containing the surfactant explains why rheograms of these dispersions had smaller thixotropic areas (1). In order to establish whether the surfactant effect could be enhanced by adding this agent to the medium prior to dispersion of the clay, dispersions of MAS were prepared to which polysorbate 80 was added, either before or after preparation. The more consistent data obtained from dispersions to which the surfactant had been added prior to MAS suggest that such dispersions are more homogeneous with respect to polysorbate 80. The results do not suggest, however, that lowering of interfacial tension during the mechanical dispersion process results in significantly greater lattice expansion or dissociation of face-to-face particle aggregates than lowering of interfacial tension subsequent to preparation of the dispersions.

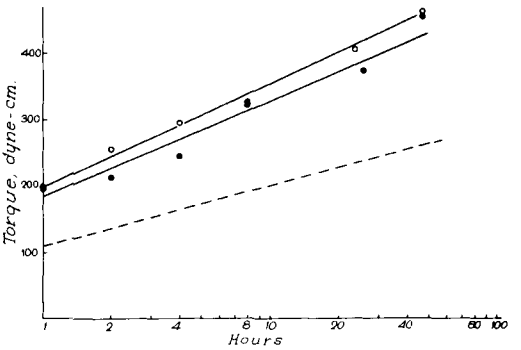


Fig. 6.—Effect of polysorbate 80 on static yield value and thixotropic recovery rate of 3% MAS suspensions. - - -, Prepared by the "cold" method without polysorbate 80; ●, 0.1% polysorbate 80 added after preparation; O, 0.1% polysorbate 80 added before preparation.

The addition of 10% zinc oxide greatly increased the static yield value of a 2.5% MAS dispersion and also increased the recovery rate (Fig. 7). The difference in static yield value is tenfold at 1 hour, and about threefold in 50 hours.[2] Zinc oxide particles are evidently capable of participating in the scaffolding structure of MAS particles in dispersion. While a possible zinc ion effect due to ion exchange cannot be excluded, it was minimized by incorporat-

[2] It must be realized that recovery rate is measured in terms of absolute increase in torque units per unit of time rather than in relative terms. Thus, a dispersion having a low initial static yield value and a low coefficient of thixotropic recovery can exhibit, nevertheless, a greater relative increase in static yield value with time than a dispersion with a higher initial static yield value as well as a higher coefficient of thixotropic recovery

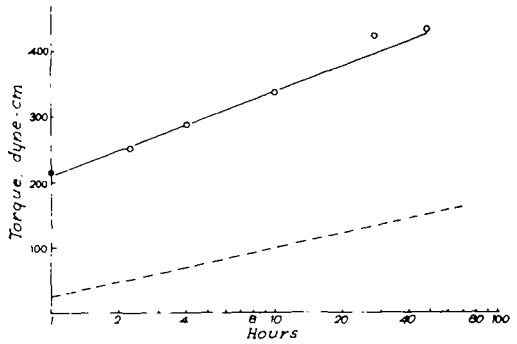


Fig. 7.—Effect of zinc oxide on static yield value and thixotropic recovery rate of 2.5% MAS dispersions. - - -, Plain suspension; O, with 10% zinc oxide.

ing zinc oxide in the aged MAS dispersions only 3 days prior to measurement. The results of this experiment may explain the lack of correlation between certain rheological properties of dispersions of two clays, bentonite and activated attapulgite, and their ability to suspend zinc oxide. Foernzler and co-workers indicate that the suspending ability of these dispersions, as reflected by the sedimentation velocity of zinc oxide, is directly related to the thixotropic area of the clay dispersion without zinc oxide (19). Wood could show subsequently, using the same data, that a similar or better correlation seems to exist between the sedimentation velocity and the reciprocal of yield value (20). Regardless of the treatment of the experimental data, however, the two clays yielded different proportionality constants and, thus, no absolute relationship of yield value or thixotropic area and suspending ability could be shown. While this may be due in part to differences in plastic viscosity as well as recovery rate of the two clays (a variable not considered in the experimental design), it is apparent from the present data that the static yield value of a pure clay dispersion differs greatly from the static yield value of a dispersion containing zinc oxide. Any attempt to correlate rheologic parameters with suspending ability should be based on the rheologic values of the final suspension, and not on those of the pure dispersion containing only the suspending agent.

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Automated Dissolution Rate Studies of Capsules and Tablets

By LOUIS C. SCHROETER and JOHN G. WAGNER

Automation of dissolution studies of solid dosage forms has been achieved through the use of a timer-controlled sampling and dilution system which automatically removes filtered samples from the dissolution apparatus, makes reproducible dilutions, and records the absorption of the diluted sample as a function of time. The U.S.P. tablet disintegration apparatus was employed to provide constant, reproducible agitation. Versatility and reliability of the apparatus has been demonstrated by comparing automated dissolution rate results with values obtained by independent, manual analyses. Design requirements and specifications of individual components have been discussed in detail so that other functionally equivalent units may be employed to achieve comparable results with those reported in this study.

Dissolution characteristics of solid dosage forms such as tablets or capsules may be determined by agitating the dosage form in a suitable fluid and periodically assaying the solution for drug content. Reproducible agitation is essential to the test. Widespread availability of the standardized U.S.P. disintegration apparatus (1) makes it ideally suited for providing constant and reproducible agitation of the tablet or capsule since both design and operation of the apparatus are exactly prescribed. Periodic withdrawal of filtered, small aliquots of test fluid provides samples containing varying percentages of dissolved drug. Ultraviolet spectrophotometry serves as a rapid

Received January 10, 1962, from the Pharmacy Research Section, Product Research and Development, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication January 29, 1962.

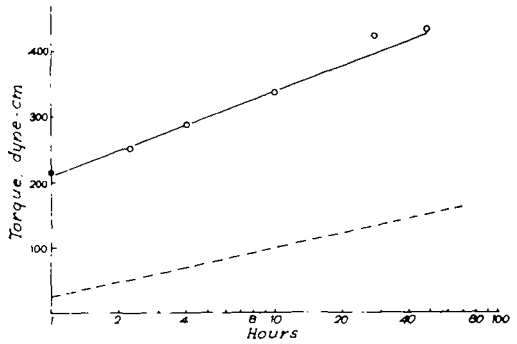


Fig. 7.—Effect of zinc oxide on static yield value and thixotropic recovery rate of 2.5% MAS dispersions. - - -, Plain suspension; O, with 10% zinc oxide.

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Received January 10, 1962, from the Pharmacy Research Section, Product Research and Development, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication January 29, 1962.

and versatile analytical method for determining drug content of most products. Assay of samples removed at various times provides the necessary data for preparing a plot of per cent drug in solution as a function of time.

Automation of the dissolution rate studies provide obvious savings in laboratory man-hours and would permit general use of this concept for designing and controlling superior solid dosage forms on a routine basis. Circulating a filtered sample of test fluid through a spectrophotometer flow cell and automatically recording the change in absorbance as drug goes into solution is the essential feature of this automated process; it provides a plot of per cent drug in solution as a function of time. The schematic diagram of such an arrangement is shown in Fig. 1. Component parts and the applications and limitations of the system will be described in detail. Emphasis will be placed on the functions performed by each component and the specifications necessary to achieve optimum versatility in operation. This permits substitution of different, but operationally equivalent, components which may be available in other laboratories.

DESCRIPTION OF THE AUTOMATED SYSTEM

Agitation of Dosage Form.—The U.S.P. disintegration apparatus and basket-rack assembly (1) provides reliable and reproducible agitation. It was used in all studies and a standard 1-L. beaker was employed as the dissolution vessel. The wire bottom of the basket was brought to within 2.3 ± 0.2 cm. of the bottom of the beaker on the downward stroke. Control of the lower limit appears to be critical since stirring action imparted to granules which have passed through the screen and rest on bottom of beaker is markedly affected by this factor. The test fluid was 37.5 ± 0.2° in all studies.

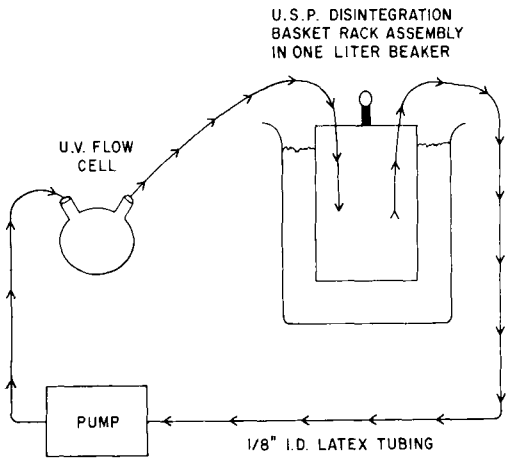


Fig. 1.—Flow pattern for continuous cycling of filtered dissolution fluid through spectrophotometer flow cell.

Filtering and Circulating the Sample.—A filtered sample of test fluid must be rapidly circulated through a spectrophotometer flow cell,[1] such as shown in Fig. 2, and returned to the beaker. Residence time of the sample in the sampling lines must be very short compared to the time required for a significant amount of drug to dissolve. Rapid pumping of the sample at variable speeds may be accomplished with the Sigmamotor model T8 pump[2] which functions by pressing, in sequence, little "fingers" of metal into the tubing, thus effecting a rapidly pulsating flow. This has the particular advantage of avoiding contamination of the fluid by the pump and also avoids hold-up of a large volume of fluid in the pump since the fluid never comes in direct contact with working parts of the pump. Small centrifugal or gear pumps constructed of stainless steel or resistant plastics also may be used if provision is made for controlling pumping rate with a rheostat.

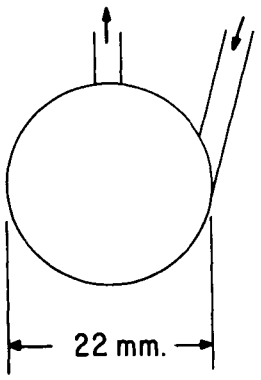


Fig. 2.—Spectrophotometer flow cell.

Filtering of the sample stream may be accomplished by placing a filter of design shown in Fig. 3 into one of the cylinders of the U.S.P. basket-rack assembly. The stainless steel filter tip is made from a 3 × 4 cm. sheet of #100 stainless steel wire cloth rolled into a cylinder with the end closed. This assembly provides reliable sampling of the solution and prevents clogging of the filter. The return stream of filtered solution should be discharged into a different cylinder, but not the cylinder containing the dosage form under test; it should not be discharged into the bottom of the dissolution beaker because this tends to magnify stirring action. Other filtering devices may be used but they should permit high flow rates so that sampling lag time is kept to a minimum. Also, it is desirable that the filter tube be weighted to prevent it from bouncing up in the cylinder and exposing the intake to air. Latex or Tygon tubing of internal diameter 1/8 inch or smaller and of shortest possible lengths should be used in the sampling line to keep sample lag time to a minimum.

[1] Ultracell Co., 22 Bland Ave., Emerson, N. J.
[2] Sigmamotor, Middleport, N. Y.

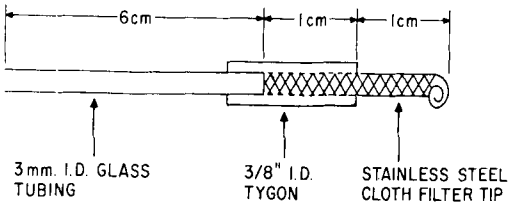


Fig. 3.—Sampling-filter device.

Spectrophotometry.—Automated measurement of the amount of drug in solution may be easily accomplished for those drugs which possess requisite chromophoric groups by automatically recording the change in absorbance of the solution flowing through the spectrophotometer flow cell. Many of the commercially available recording spectrophotometers, such as the Cary model 11, can be set to record absorption at a given wavelength (usually at a point where drug molecule exhibits maximum absorption) as a function of time. This requires no modification of the recording spectrophotometer other than arranging the cell compartment cover to permit entrance of tubing to the flow cell. The recording spectrophotometer may be converted to regular use in less than 30 seconds. However, as a permanent arrangement, the use of such a recording spectrophotometer would commit a versatile and expensive instrument to a specific job function. For this reason, a Beckman model DU spectrophotometer was converted for this specialized purpose.

Conversion of the Beckman DU spectrophotometer to an instrument which will record absorbance as a function of time may be accomplished with the Gilford model 200 optical density converter.[3] This attachment consists of two parts: a photodetector which is fastened to the spectrophotometer in place of the standard detector; and an electronic power supply and converter-amplifier. No modification of the spectrophotometer is necessary; the conversion can be made in minutes and does not impair use of the spectrophotometer for manual, conventional operation. The Beckman DU instrument was chosen for its excellent monochromator system; however, any spectrophotometer having a similar monochromator covering the ultraviolet and visible spectrum from 220 to 800 mμ and having a stabilized light source can be used. Recording can be done on any self-balancing potentiometer recorder with a 50 mv. full-scale sensitivity and a response time of one-half second.

Recording.—A Texas Instrument Servariter recorder model PWS[4] was used in all studies. This recorder has a chart speed selector which permits rapid selection of 10 different chart speeds. Normal range of chart speeds in most studies varied from $^1/_4$ to 2 in. per min.

Intermittent Sampling and Dilution.—Increased versatility of an automated dissolution device may be accomplished with the use of a sampling dilution arrangement shown schematically in Fig. 4. Introduction of a three-way magnetic valve[5] into the sample stream serves as a means of diverting a fixed, small volume of the sample stream into a diluent stream. The valve should have a minimum orifice of $^1/_{16}$ inch so that sample retention is reduced to a minimum. The valve is actuated by a signal from a timer which permits preselection of sampling time and duration of sample stream diversion. Thus, if the flow rate in the sample stream is 1 ml. sec.$^{-1}$, opening the valve for one-half second removes 0.5 ml. of sample. Withdrawal of 10 samples of 0.5-ml. volume from 1 L. of fluid during the course of a dissolution study introduces an overall error of only 0.5%.

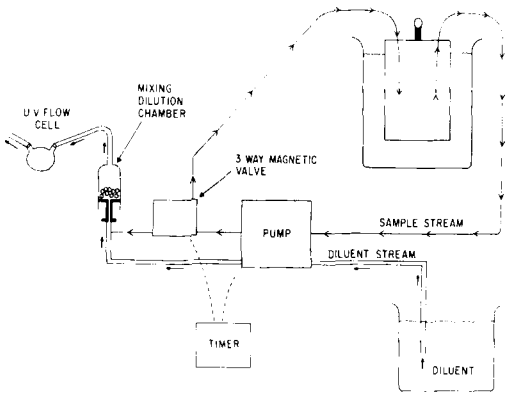


Fig. 4.—Flow pattern of filtered solution when intermittent sampling with dilution employed.

Transport of the diverted sample to the spectrophotometer flow cell may be accomplished by injecting it into a diluent stream flowing at a constant rate. However, streamline flow does not serve for effective mixing (2, 3). Mixing the sample and diluent stream may be accomplished by using a turbulent flow mixing chamber (Fig. 5). It is desirable to have a variable volume mixing chamber to provide requisite control of effective dilution necessary in adjusting absorbances of diluted sample stream between 0.1 and 3.0. It may be advan-

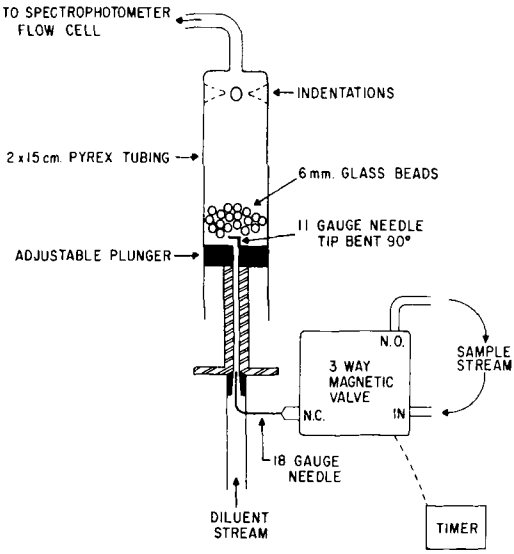


Fig. 5.—Three-way magnetic valve and turbulent flow dilution-mixing chamber for intermittent sampling and dilution of sample stream.

[3] Gilford Instrument Laboratories, Inc., Oberlin, Ohio.
[4] Texas Instruments, Inc., Houston, Tex.
[5] Skinner Electric Valve Div., New Britain, Conn.

tageous to employ fixed volume mixing chambers for studying a series of tablets containing the same amount of drug, *i.e.*, in cases where dilution of sample stream remains constant. Fixed volume mixing chambers can be constructed from ordinary volumetric pipets by cutting off the tubing to within 1 cm. of the body and filling the body with 3- or 4-mm. glass beads. Most reproducible mixing occurs when these are employed in a vertical position with sample and diluent entering the bottom and emerging mixed from the top.

Controlled flow of diluent may be accomplished with a hydrostatic device constructed from a 500-ml. separator modified with a side arm tube (about 5 cm. from top) for overflow. Adjusting height of liquid level permits accurate control of diluent flow. The Sigmamotor pump described above can also be used to pump both the sample stream and the diluent stream simultaneously since it will accommodate tubing on both sides of the "fingers." Different tension adjustments on either side of the pump and the use of larger diameter tubing for the diluent stream permits variable pumping rate selections for both streams. It is not necessary to know the exact dilution factor since all measurements during a dissolution study are relative. However, effective dilution must be reproducible during the course of the study and for comparative studies. Flow rate control may be achieved by periodically checking both the sample and diluent stream with a flow meter.[6]

Timing sequence may be provided by any timer or series of timers which will, at predetermined intervals of from $^1/_2$ to 5 minutes, open for a period from $^1/_2$ to 5 seconds, and then close the three-way solenoid valve. The timer should then automatically reset (recycle) and repeat the sequence. A sequential timer[7] was used to control the operation since it contained six circuits; however, other, less sophisticated timers would be equally satisfactory if they fulfilled the timing requirements. If a Sigmamotor pump is used for pumping both sample and diluent stream, it may be advisable to conserve the diluent, especially when buffers or other color-developing solutions are employed.

Conservation of diluent fluid may be accomplished by intermittent timer-controlled operation of the pumping system. A multiple circuit timer should control the following sequence: (*a*) pump on, 15–20 sec.; (*b*) valve open, $^1/_2$–5 sec.; (*c*) valve shut, 15–30 sec.; (*d*) pump off, 60–300 sec.; (*e*) repeat cycle. Operating the pump for a period of at least five residence times (15 sec.) permits flushing of lines and taking a representative sample from the dissolution vessel. The pump must run a minimum of 15 to 30 sec. after sampling to flush dilution-mixing chamber and spectrophotometer flow cell.

RESULTS AND DISCUSSION

Continuous Monitoring of Dissolution Process.—The schematic arrangement of equipment shown in Fig. 1 was used to follow the dissolution process of a 4-mg. steroid tablet. Complete dissolution of one tablet in 750 ml. of pH 1.35 buffer yielded a solution with an absorbance of 0.21. This low absorbance of the dissolution fluid provided a good test of the sensitivity of the automated procedure for determining dissolution characteristics of dosage forms containing small amounts of drug or drugs with low molar absorptivities. Total volume of sampling lines, filter device, and flow cell was 7.3 ml.; the sample flow rate was 1.34 ml. sec.$^{-1}$. Residence time in the sampling system was 5.4 sec. This can be reduced further by decreasing the volume of the sampling system or increasing flow rate, or both; however, these are upper limits on permissible flow rates. If the flow rate is greater than 2 ml. sec.$^{-1}$, this tends to contribute to the stirring action in the dissolution beaker and results in faster dissolution of the tablet or capsule. A filtered sample stream from the beaker containing drug from one tablet wholly dissolved (100% drug in solution) was pumped through the flow cell and the recorded response adjusted to read 100 units or 100% drug in solution. Sample lines were flushed with pH 1.35 buffer and the test repeated by placing one tablet in the U.S.P. basket-rack assembly immersed in a new beaker containing 750 ml. of pH 1.35 (μ = 0.1) buffer at 37.5°. The dissolution curve produced by the automated procedure is shown in Fig. 6 along with 2 independent individual dissolution rate determinations of other tablets from the same lot.

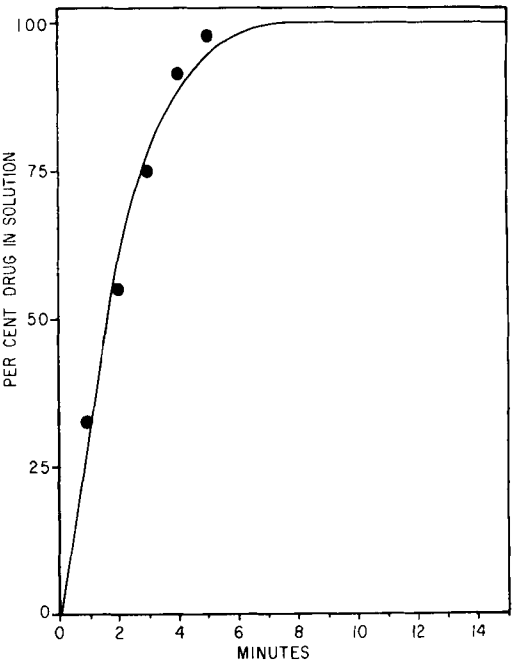


Fig. 6.—Dissolution characteristics of a steroid tablet in 750 ml. buffer (μ 0.1) at 37.5° using U.S.P. disintegration apparatus without disks. Solid line redrawn from curve obtained using automated procedure. Solid circles represent independent assays of tablets from same lot.

The schematic arrangement shown in Fig. 1 serves very well for the accurate monitoring of the dissolution process of this particular steroid tablet. However, it must be emphasized that not all dosage forms contain just the right amount of drug which, when dissolved in about 1 L. of fluid, will yield solutions having absorbance values between 0.1

[6] Flowrator, Fischer and Porter Co., Hatboro, Pa.
[7] Multiflex, Eagle Timer Corp., Moline, Ill.

and 3.0. Many dosage forms contain drugs with high molar absorptivities so that resultant solutions from complete dissolution of the drug have absorbance values of 10 or higher. Thus, with the total volume of fluid set at about 1 L. and the limit of spectrophotometric sensitivity fixed at 0.1 to 3.0 absorbance units, the need for dilution of sample stream becomes evident. The sample stream, once it is diluted, cannot be returned to the test beaker because this would increase the total volume of fluid present. Likewise, the dissolution beaker cannot be constantly sampled nor can the combined withdrawn samples constitute a large volume because this would deplete the fluid in the beaker.

Sampling with Dilution.—A typical "combination of ingredients" tablet containing aspirin, phenacetin, and caffeine was selected to demonstrate automated sampling with dilution. One tablet was dissolved completely in 750 ml. of pH 1.35 buffer (μ = 0.1) by equilibrating at 37.5° for 1 hour. Absorbance of the undiluted solution was 23.6 at the wavelength of maximum absorption which was a composite of the 3 chromophores. This indicated the need for an effective dilution of 20× to give a measurable absorbance. Sample flow rate was regulated to 1 ml. sec.$^{-1}$ and the timer adjusted to send a $^1/_2$ sec. signal (0.5 ml. sample) to the three-way solenoid valve every 1.7 min. Volume of the sampling line and valve was 4.1 ml.; sample lag time was 4.1 sec. The wavelength of the absorption maximum (230 mμ) of the dissolved mixture in pH 1.35 buffer was selected on the monochromator. The recorder was then balanced to give a maximum response to 100 units (100% drug in solution) by manipulating the flow rate of pH 1.35 diluent and adjusting the turbulent flow mixing chamber volume. The sampling system was flushed with pH 1.35 buffer and connected with a new beaker containing 750 ml. of pH 1.35 (μ = 0.1) maintained at 37.5°. One tablet was placed into the U.S.P. disintegration basket assembly and immersed into the buffer solution and agitated at the prescribed rate of 30 cycles per min. Introduction of the tablet into the buffer marked zero time for the process. Reproduction of the curve produced by the recorder is shown in Fig. 7. The heights of the solid vertical lines represent instrumental response or per cent drug in solution. Absorbance of the solution flowing through the cell rapidly returns to zero as the diluted sample is flushed from the cell. The one flow cell serves as a sample cell and as a blank cell since the diluent stream constantly flushes the cell; this assures instrumental reliability of measurements made over long periods. The inherent reliability of the automated system is shown by the close fit of peak heights obtained with the automated procedure to the individual points obtained by an independent method on tablets from the same lot.

Intermittent Circulation with Sampling and Dilution.—A sulfonylurea tablet formulation was used to evaluate the applicability of intermittent fluid circulation followed by sampling with dilution. Two tablets of the sulfonylurea product were dissolved in 750 ml. of pH 7.2 THAM[8] buffer by equilibrating with stirring at 37.5° for 1 hour. Complete solution of the drug content of the tablets corresponds to 100% drug in solution or 1000 mg. of drug dissolved in 750 ml. of buffer. Absorbance of the undiluted solution was 65 at the wavelength of maximum absorption, thus indicating the need for an effective dilution of from 30 to 50×. The sample flow rate was regulated at 1.0 ml. sec.$^{-1}$ and the timer adjusted to start the pump 15 sec. before sending a signal to the valve and to continue pumping 15 sec. after the event. The timer was adjusted to send a 0.5-sec. (0.5-ml. sample) signal to the three-way magnetic valve every 60 sec. Volume of sampling lines and valve was 3.5 ml.; the time required for a sample to complete the circuit from beaker and return was 3.5 sec. Monochromator was adjusted to provide monochromatic radiation at the absorption maximum of the sulfonylurea in pH 7.2 THAM buffer (228 mμ). The recorder was balanced to give a maximum response of 100 units (arbitrarily marked as 1000 mg.) by manipulating the flow rate of diluent and adjusting volume of the mixing chamber. The sampling system was flushed with fresh pH 7.2 THAM buffer and connected with a new beaker containing 750 ml. of pH 7.2 THAM buffer maintained at 37.5°. Two tablets were placed into the U.S.P. disintegration basket-rack assembly and immersed into the buffer solution; this marked zero time on the recorder. Reproduction of the curve obtained from the recorder is shown in Fig. 8. The heights of the solid vertical lines represent instrumental response or per cent drug in solution. Absorbance of the solution rapidly returns to zero as the diluent sample passes through the cell; this serves as a further in-process instrumental check. Analyses of solution composition at various times during the dissolution test by an independent spectrophotometric method served to validate the method.

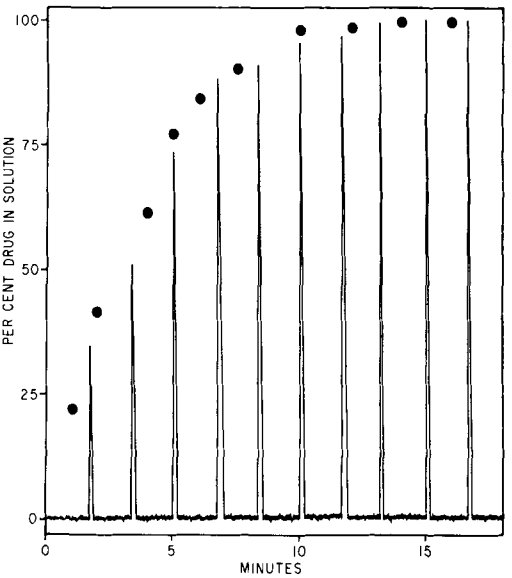


Fig. 7.—Dissolution profile of acetophenetidin-acetylsalicylic acid-caffeine tablet in 750 ml. pH 1.35 buffer (μ 0.1) using U.S.P. disintegration apparatus without disks. Vertical solid lines redrawn from curve obtained using automated procedure with dilution device. Solid circles represent independent assays of tablets from the same lot.

[8] Tris(hydroxymethyl)aminomethane 0.1 *N* in water brought to pH 7.2 by addition of hydrochloric acid.

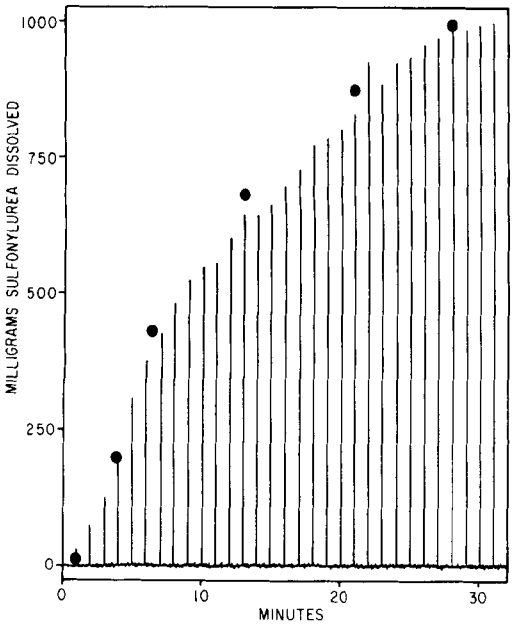


Fig. 8.—Dissolution profile of two sulfonylurea tablets in 750 ml. pH 7.2 THAM buffer at 37.5° using U.S.P. disintegration apparatus without disks. Vertical solid lines redrawn from curve obtained using automated procedure with dilution device. Theoretical drug content of two test tablets taken as 1000 mg. Solid circles represent values obtained by simultaneous, independent assays of dissolution fluid.

CONCLUSIONS

Automated dissolution rate studies of solid dosage forms serve as an extremely useful method for controlling the quality of these products. The method is applicable to the evaluation of virtually all tablets, capsules, and granules containing drugs soluble in about 1 L. of dissolution fluid. The total dissolution profile expressed as per cent drug in solution as a function of time may be obtained for these products. However, dosage forms containing drugs not wholly soluble in the dissolution medium may also be evaluated; results are expressed in terms of absolute initial rate of dissolution.

Savings in laboratory man-hours can be enormous if the automated procedure is used regularly to control the quality of solid dosage forms. One investigator working with the automated device can perform the same number of dissolution studies as four technicians working with conventional, manual procedures. Also, the automated procedure uses a modified Beckman DU spectrophotometer in place of a Cary recording spectrophotometer for measuring absorption changes in the solution. This frees the more expensive recording spectrophotometer for other more versatile analytical jobs. The automated device also serves as a useful and versatile analytical tool for automatically re-recording the progress of chemical kinetic reactions which involve changes in absorption of monochromatic light.

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(1) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 934.
(2) Dryden, H. L., *Ind. Eng. Chem.*, **31**, 416(1939).
(3) Levenspiel, O., *ibid.*, **50**, 343(1958).

Synthesis and Antifungal Evaluation of Some Derivatives of Dialkoxybenzoic and Dialkoxycinnamic Acids

By A. K. KAPADIA† and GAIL A. WIESE

A number of dialkylaminoalkyl esters of dialkoxybenzoic and dialkoxycinnamic acids and their halogenated analogs were prepared. The compounds, in the form of their hydrochloride salts, together with the parent acids were tested *in vitro* against three pathogenic fungi using undecylenic acid and griseofulvin as controls. In general, the results of the tests indicated that most of the compounds had little or no antifungal activity *in vitro*.

IT HAS BEEN shown that fungal diseases in man are a growing health hazard (1). The search for antifungal agents has been in progress for several decades. In reviewing the literature it was noted that there have been few investigations directed toward the study of antifungal activity of the derivatives of dialkoxybenzoic and dialkoxycinnamic acids and their halogenated analogs.

Received January 29, 1962, from the College of Pharmacy, State University of Iowa, Iowa City.
Accepted for publication March 1, 1962.
Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by A. K. Kapadia in partial fulfillment of the Ph.D. degree requirements.
† Recipient of honorable mention in the Lunsford Richardson Award, Pharmacy 1962. Present address: Haffkine Institute, Bombay 12, India.

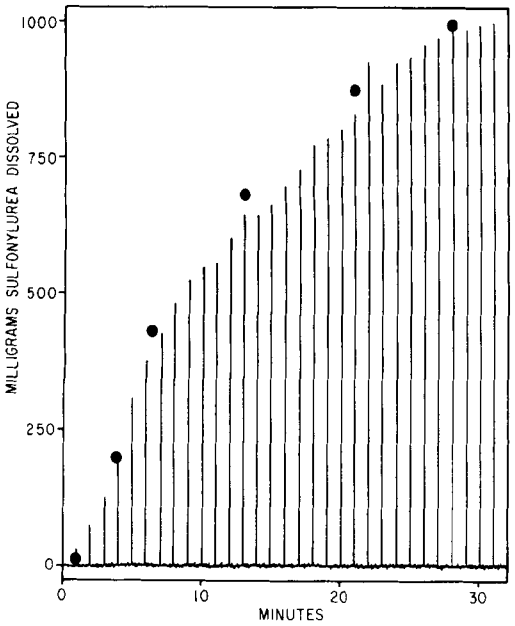


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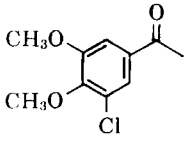
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† Recipient of honorable mention in the Lunsford Richardson Award, Pharmacy 1962. Present address: Haffkine Institute, Bombay 12, India.

TABLE 1.—COMPOUNDS SYNTHESIZED AND/OR TESTED

No.	Name	Yield, %	M.p.,[a] °C. Observed	M.p.,[a] °C. Reported	Ref.
I	Benzoic acid[b]	...	...	...	...
II	Vanillic acid	74.0	210–211	210–211	8
III	Veratric acid[b]	56.6	180–181	180–181	9
IV	5-Chlorovanillin	83.5	165–166	165–166	10
V	5-Chlorovanillic acid	80.0	242	242	11
VI	5-Chloroveratric acid[b]	70.0	189–190	189–190	12
VII	5-Bromovanillin	95.5	164	164	13
VIII	5-Bromovanillic acid	72.5	231–232	231–232	14
IX	5-Bromoveratric acid[b]	100.0	192–193	192–193	15
X	5-Iodovanillin	78.6	179–180	179–180	16
XI	5-Iodoveratraldehyde	77.2	72–73	72–73	12
XII	5-Iodoveratric acid[b]	80.4	184–185	184–185	12
XIII	5-Chloro-4-hydroxy-3-ethoxybenzaldehyde	80.0	149–150	...	[c]
XIV	5-Chloro-4-hydroxy-3-ethoxybenzoic acid	100.0	199–200	...	[d]
XV	5-Chloro-4-methoxy-3-ethoxybenzoic acid[b]	58.7	167–168	...	[e]
XVI	3-Ethoxy-4-hydroxybenzoic acid	66.0	164–165	164–165	17
XVII	5-Bromo-4-hydroxy-3-ethoxybenzoic acid	79.0	207	207	17
XVIII	5-Bromo-4-methoxy-3-ethoxybenzoic acid[b]	79.0	183–184	183–184	17
XIX	Cinnamic acid[b]	...	...	...	...
XX	3-Ethoxy-4-acetoxycinnamic acid	45.2	176–177	...	...
XXI	5-Chloroferulic acid	83.0	251	251	18
XXII	5-Chloro-3,4-dimethoxycinnamic acid[b]	80.2	126–127	126–127	19
XXIII	5-Bromoferulic acid	100.0	257–258	257–258	20
XXIV	5-Bromo-3,4-dimethoxycinnamic acid[b]	88.0	138–139	138–139	21

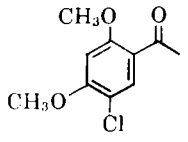
[a] Melting points are uncorrected and were taken on Fisher-Johns melting point apparatus. [b] Tested for antifungal activity. [c] *Anal.*—Calcd. for $C_9H_9ClO_3$: Cl, 17.7. Found: Cl, 17.55. [d] *Anal.*—Calcd. for $C_9H_9ClO_4$: Cl, 16.4. Found: Cl, 16.34. [e] *Anal.*—Calcd. for $C_{10}H_{11}ClO_4$: Cl, 15.4. Found: Cl, 15.42.

It is interesting to note that the structural fragment I included in this investigation is a



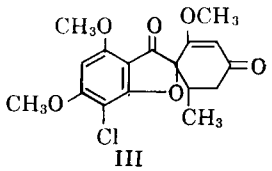
I

position isomer of the structure II present in the



II

griseofulvin molecule III.

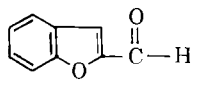


III

MacMillan has shown that the aromatic chlorine substituent in the griseofulvin molecule enhances the antifungal activity of griseofulvin (2).

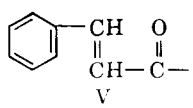
Woodward, *et al.* (3), have studied the fungicidal properties of various halogens and alkyl substituents on the aromatic ring of phenol *in vitro*. They found that in going from chlorine to bromine to iodine, there was a four to tenfold increase in antifungal activity. Taking these facts into consideration, it was decided that the effect of removal of halogen from the aromatic ring and the interchange of chlorine for bromine and iodine should be studied.

Pan and Wiese (4) found that benzofuran-2-carboxaldehyde (IV) had



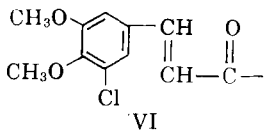
IV

significant antifungal activity as compared to undecylenic acid *in vitro*. The cinnamoyl group (V) is present in benzofuran-2-carboxaldehyde (IV).



V

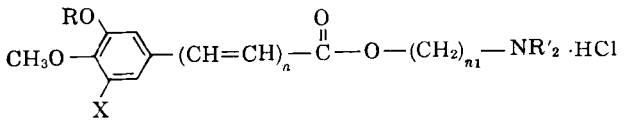
The structural fragment VI was included in this investigation. This is a vinylog of I.



VI

Since dialkylaminoalkyl esters of other acids have shown useful properties (5–8), the dialkylaminoalkyl esters were of interest in this investigation. These alkamine esters were water insoluble, therefore they were converted into the hydrochloride salts. The compounds prepared in this investigation are summarized in Tables I and II.

TABLE II.—HYDROCHLORIDES OF DIALKYLAMINOALKYL ESTERS SYNTHESIZED AND TESTED



No.	R	X	n	m	R′	Yield, %	M.P.,[a] °C.	Formula[b]	Analysis, % N[c] Calcd.	N[c] Found	Halogen Calcd.	Halogen Found
XXV	CH_3	H	0	2	CH_3	10.0	165–166	$C_{13}H_{20}ClNO_4$	4.84	4.86	12.26	12.10
XXVI	CH_3	H	0	2	C_2H_5	37.8	145–146	$C_{15}H_{24}ClNO_4$	4.41	4.34	11.18	11.39
XXVII	CH_3	Cl	0	2	CH_3	73.7	152–153	$C_{13}H_{19}Cl_2NO_4$	4.32	4.35	21.92	22.20
XXVIII	CH_3	Cl	0	[d]	CH_3	44.8	132–133	$C_{14}H_{21}Cl_2NO_4$	4.14	4.19	21.00	20.93
XXIX	CH_3	Cl	0	2	C_2H_5	32.0	154–155	$C_{15}H_{23}Cl_2NO_4$	3.98	3.98	20.18	20.18
XXX	CH_3	Cl	0	3	CH_3	51.9	135–136	$C_{14}H_{21}Cl_2NO_4$	4.14	4.10	21.00	21.05
XXXI	CH_3	Cl	0	3	C_2H_5	49.7	140–141	$C_{16}H_{25}Cl_2NO_4$	3.83	3.86	19.40	19.20
XXXII	CH_3	Br	0	2	CH_3	63.7	161–162	$C_{13}H_{19}BrClNO_4$	3.80	3.75	31.34	31.00
XXXIII	CH_3	Br	0	2	C_2H_5	48.7	164–165	$C_{15}H_{23}BrClNO_4$	3.53	3.60	29.14	28.70
XXXIV	CH_3	I	0	2	C_2H_5	60.4	166–167	$C_{15}H_{23}ClINO_4$	3.16	3.20	36.64	37.20
XXXV	C_2H_5	Cl	0	2	CH_3	35.6	112–113	$C_{14}H_{21}Cl_2NO_4$	4.16	4.22	20.77	21.00
XXXVI	C_2H_5	Cl	0	2	C_2H_5	39.1	133–134	$C_{16}H_{25}Cl_2NO_4$	3.84	3.88	19.18	19.22
XXXVII	C_2H_5	Br	0	2	CH_3	28.7	109–110	$C_{14}H_{21}BrClNO_4$	3.66	3.70	30.19	30.24
XXXVIII	C_2H_5	Br	0	2	C_2H_5	28.1	130–131	$C_{16}H_{25}BrClNO_4$	3.41	3.45	28.14	28.30
XXXIX[e]	C_2H_5	H	1	2	CH_3	26.2	151–152	$C_{17}H_{23}ClNO_5$	3.92	3.85	9.93	9.85
XL[e]	C_2H_5	H	1	2	C_2H_5	39.7	175–176	$C_{19}H_{28}ClNO_5$	3.62	3.57	9.20	9.12
XLI	CH_3	Cl	1	2	CH_3	25.6	140–141	$C_{15}H_{21}Cl_2NO_4$	4.00	4.10	20.29	19.95
XLII	CH_3	Cl	1	2	C_2H_5	28.9	148–149	$C_{17}H_{25}Cl_2NO_4$	3.70	3.69	18.78	19.00
XLIII	CH_3	Br	1	2	C_2H_5	30.6	149–150	$C_{17}H_{25}BrClNO_4$	3.32	3.26	27.34	27.00

[a] Melting points are uncorrected and were taken on Fisher-Johns melting point apparatus. [b] All the compounds were recrystallized from an absolute alcohol-anhydrous ether mixture. [c] Nitrogen analysis by a semimicro Kjeldahl method. [d] $-(CH_2)-$ is equal to $-\underset{\displaystyle CH_3}{\overset{|}{CH}}-CH_2-$. [e] $p-OCH_3$ is replaced by $p-OCOCH_3$.

TABLE III.—MINIMUM WIDTH OF ZONES OF INHIBITION[a]

Compound No.[b]	*T. mentagrophytes*, mm. Concn. % w/v 0.5	1.0	2.0	*T. rubrum*, mm. Concn. % w/v 0.5	1.0	2.0	*M. gypseum*, mm. Concn. % w/v 0.5	1.0	2.0
I	3	3	3	3	5	6	0	0	2
III	0	1	2	1	2	4	0	0	0
VI	0	1	3	1	2	2	0	0	0
IX	0	0	0	1	4	4	0	0	0
XV	0	0	0	1	2	3	0	0	0
XIX	4	5	10	5	11	13	2	4	5
XXI	4	4	4	2	3	5	0	0	0
XXIV	2	2	2	2	2	3	0	0	0
XXX	0	1	2	1	2	3	0	1	2
XXXI	2	2	3	2	2	2	1	2	2
XXXVI	2	2	3	2	2	2	0	1	2
XLI	2	2	3	1	1	2	0	2	3
XLIII	2	4	10	1	2	2	1	2	2
Undecylenic acid[c]	10	10	11	9	9	9	3	5	5
Ethyl vanillate[c]	0	8	19	2	4	16	2	8	13
Ethanol 95%[d]	0	0	0	0	0	0	0	0	0
Griseofulvin[e]		14			1			7	

[a] Average of two measurements. [b] Numbers according to Tables I and II. [c] Controls. [d] Solvent. [e] Saturated solution.

ANTIFUNGAL EVALUATION

The compounds were tested *in vitro* against *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Microsporum gypseum*. The testing procedure was similar to that described by Waters (22), with the exceptions that ethanolic solutions of the compounds and a 7-day incubation period were employed. At the end of the incubation period, the minimum zone of inhibition was measured. The zone of inhibition was the distance between the periphery of the paper disk and the colony growth. Undecylenic acid and griseofulvin were used as controls. Ethyl vanillate and 95% ethanol were also included in this study. The results of this antifungal study are given in Table III. Each value in this table represents an average of two zones of inhibition. Only those compounds which had a zone of inhibition 3 mm. and greater are reported in Table III.

DISCUSSION

The dialkylaminoalcohols used in these studies are readily available. The required acids were synthesized according to the procedures described in the literature with certain modifications.

Raiford and co-workers (12, 14, 15) have reported the synthesis of a number of dialkoxybenzoic acids. Vanillin was first alkylated and subsequently oxidized to the acid with potassium permanganate. We found that oxidizing vanillin to the corresponding vanillic acid and then alkylating it was a more efficient process and gave better yields.

The method of Pearl (8) for preparing vanillic acid (II) from vanillin using silver oxide in an alkaline medium was preferred for the preparation of 5-chlorovanillic acid (V), 5-bromovanillic acid (VIII), and 5-chloro-4-hydroxy-3-ethoxybenzoic acid (XIV).

Veratric acid (III) was synthesized from vanillic acid (II) according to the method used by Fosdick and Starke (23) for preparing 3,4-dimethoxycinnamic acid from ferulic acid. The same procedures were used for preparing 5-chloroveratric acid (VI), 5-bromoveratric acid (IX), and 5-chloro-4-methoxy-3-ethoxybenzoic acid (XV) from their respective aldehydes. Attempts to prepare 5-iodoveratric acid (XII) in this way were unsuccessful. Therefore, the following method was employed: the aldehyde, 5-iodovanillin was first methylated to give 5-iodoveratraldehyde (XI) which was subsequently oxidized to 5-iodoveratric acid (XII) with potassium permanganate. In the case of 5-bromo-4-methoxy-3-ethoxybenzoic acid (XVIII), 3-ethoxy-4-hydroxybenzaldehyde was first oxidized to 3-ethoxy-4-hydroxybenzoic acid (XVI). This compound was brominated yielding 5-bromo-4-hydroxy-3-ethoxybenzoic acid (XVII). Subsequent methylation gave rise to 5-bromo-4-methoxy-3-ethoxybenzoic acid (XVIII).

The compounds, 5-chloro-3,4-dimethoxycinnamic acid (XXI) and 5-bromo-3,4-dimethoxycinnamic acid (XXIV) were synthesized according to the methods of Pearl and Beyer (18) and Fosdick and Starke (23).

The aldehydes, 5-chlorovanillin (IV) and 5-chloro-4-hydroxy-3-ethoxybenzaldehyde (XIII), were obtained by a modification of the method of Menke and Bentley (10). Because of solubility problems, carbon tetrachloride was employed as a solvent instead of chloroform. The compounds, 5-bromovanillin (VII) and 5-iodovanillin (X) were obtained by the methods of McIvor and Pepper (24) and Erdtman (16), respectively.

The alkamine esters were prepared by refluxing the dialkylaminoalcohol in dry benzene with the appropriate acid chloride. The alkamine esters that formed precipitated as the hydrochloride salts.

The results of the antifungal studies, as shown in Table III, indicate that none of the compounds had an antifungal activity comparable to that of the compounds used as controls.

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Thin-Layer Chromatography in Drug Analysis I

Identification Procedure for Various Sulfonamides in Pharmaceutical Combinations

By SHELDON KLEIN† and B. T. KHO

A thin-layer chromatographic procedure is described for identifying various sulfonamides when present in pharmaceutical combinations. Successful identification has been accomplished for sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, sulfanilamide, sulfaquinoxaline, and sodium sulfabromomethazine. The critical effect of water in the solvent system and the use of various detection methods, including fluorescent plates and Ehrlich's and Bratton-Marshall's reagent sprays are described. The procedure can be completed within 2 hours.

SINCE the introduction of thin-layer chromatography (TLC) into pharmaceutical literature by Stahl (1), numerous articles have been published pertaining to drug analysis by use of this technique (2–9). The advantage of TLC over paper chromatography is its speed and ability to withstand corrosive substances for use as spraying agents, such as sulfuric acid for the determination of meprobamate (2). A disadvantage is the difficulty in obtaining reproducible R_f values. This, however, can be overcome by use of standards along with the unknown samples.

The U.S.P. XVI (10) uses a precipitation procedure for its identification of a trisulfapyrimidine combination of sulfadiazine, sulfamerazine, and sulfamethazine. The N.F. XI (11) uses a paper chromatographic separation for the identification of sulfadiazine and sulfamerazine in the presence or absence of sulfacetamide. These are time consuming and tedious to perform. The TLC procedure described in this paper can be done within 2 hours. Wollish, Schmall, and Hawrylyshyn (8) reported a TLC method and approximate R_f values for sulfanilic acid, sulfadiazine, N[1]-(3,4-dimethyl-5-isoxazoyl) sulfanilamide and its acetyl derivative,[1] and 2,4 - dimethoxy - 6 - sulfanilamido - 1,3 - diazine.[2]

The purpose of this paper is to provide, by means of TLC, a relatively rapid systematic separation of commercially available sulfonamide combinations. Using a modification of the solvent system described by Wollish, *et al.* (8), separation and identification of various sulfonamides was accomplished. The influence of the amount of water on the successful separation has also been studied.

EXPERIMENTAL

Primary Preparation of Plates.—The Stahl apparatus[3] and method were used for the preparation of the chromatoplates. Silica gel G,[4] 30 Gm., was placed in a dry 8-oz. mortar.

Preparation of Neutral Plates.—Distilled water, 40 ml., was added while stirring slowly. When the entire mass was of a uniform consistency, an additional 20 ml. of distilled water was added, and the mixture was stirred until uniform.

Preparation of Fluorescent Plates.—Fluorescein, 20 mg., was dissolved in 1.2 ml. of 0.1 *N* sodium hydroxide. Sufficient distilled water was added to make 60 ml. The preparation was then the same as described for the neutral plates, using the fluorescein solution instead of water.

The total stirring time should not exceed 1.5 minutes. The suspension was poured into the applicator and spread immediately over five plates (200 × 200 mm.) or 20 strips (200 × 50 mm.). After air drying for approximately 5 minutes to allow evaporation of excess water, the plates were stored in a desiccator over silica gel. Before use, they were activated at 105° for 30 minutes and set aside to cool. Starting points were marked along an imaginary line 15 mm. from one edge, and a finishing line was drawn 150 mm. from the start.

Standards.—A 10-mg. quantity of each sulfonamide was dissolved in separate 50-ml. portions of acetone. Sulfonamides used were: sulfadiazine (I), sulfamerazine (II), sulfamethazine (III), sulfathiazole (IV), sulfacetamide (V), sulfanilamide (VI), sulfaquinoxaline (VII), and sodium sulfabromomethazine (VIII).

Received February 26, 1962, from the Quality Control Section, Merck Sharp & Dohme, Division of Merck & Co., Inc., Philadelphia, Pa.

Accepted for publication March 8, 1962.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

The cooperation and encouragement of Dr. L. Paul Sinotte and Mr. Theodore P. Michaels in carrying out this project are gratefully acknowledged. The authors also wish to thank the Technical Services Unit for helpful suggestions, Mr. Karl Wrightman for photography, and Miss Kathleen Smith for her secretarial assistance.

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[1] Marketed as Gantrisin and Gantrisin acetyl by Hoffmann-LaRoche.

[2] Marketed as Madribon by Hoffmann-LaRoche.

[3] Obtainable from C. Desaga, Heidelberg, Germany, Importer, Brinkmann Instruments Inc., Great Neck, L. I., N. Y.

[4] A standardized mixture of silicic acid and calcium sulfate obtainable from E. Merck, Darmstadt, West Germany; Importers, Brinkmann Instruments Inc., Great Neck, L. I., N. Y.; Terra Chemicals Inc., 500 Fifth Ave., New York 36, N. Y.

Tablet Samples.—A quantity containing approximately 10 mg. of the individual sulfonamide in the largest concentration was added to a 125-ml. glass-stoppered Erlenmeyer flask and extracted with 50 ml. of acetone.

Suspension Samples.—The extraction procedure was the same as that for the tablets except where the products contained interfering ingredients. To these latter samples, 1 ml. of distilled water was added before shaking with the acetone. Cloudy solutions were centrifuged before applying a sample to the plate.

Quantity of Sulfonamide Applied.—A 1-mcg. quantity of sulfonamide was applied to the chromatoplates.

Primary Solvent System.—Chloroform-ethanol-heptane (1:1:1 plus an amount of water depending upon the sulfonamide combination). It was prepared by mixing 66 ml. each of reagent grade chloroform, anhydrous ethanol, and heptane. Anhydrous sodium sulfate was added and the mixture shaken. Before using, sufficient water was added to the decanted solvent to obtain the optimum water percentage. After the addition of water, the level was checked using Karl Fisher reagent.

Concentration of Water.—Table I shows the amounts of water used for various mixtures. Slight variances from the values shown altered the chromatoplates sufficiently to reduce the sensitivity of the procedure.

TABLE I.—AMOUNT OF WATER ADDED TO THE PRIMARY SOLVENT

	Suspected Sulfonamides	Water,[a] %
A	I, II, III, VII, VIII	1.0–1.8 (1.5 optimum)
B	IV	1.2
C	V	1.5–1.8
D	VI	1.5

[a] Per cent water for sulfonamides in A was used when no others were suspected. B, C, and D were used when these sulfonamides appeared in addition to any of those in A.

Spray Reagents.—Aqueous hydrochloric acid, 1*N*, 5% aqueous sodium nitrite, 0.1% alcoholic N-(1-naphthyl)ethylenediamine dihydrochloride (Bratton-Marshall reagent) were used as spray reagents.

Distance Developed.—The chromatoplates were developed over a distance of 150 mm.

Method for Neutral Plates (A).—Samples (5 μl.) and standard solutions (5 μl.) representing each sulfonamide theoretically present in the samples were applied to the plates and dried by a current of air. The developing tanks were allowed to equilibrate at least 3 hours before inserting the chromatoplate. Up to 18 individual samples were applied per plate. When development of 150 mm. was completed, the plates were removed, air dried, and sprayed with 1 *N* hydrochloric acid followed by 5% sodium nitrite.

Any yellow spots representing sulfathiazole or sulfaquinoxaline were marked. The plates were then dried at 100° (usually for less than 5 minutes) until free from excess nitrous acid (when no yellow areas other than those marked could be seen). Coupling with 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride produced reddish-purple spots which were marked and the R_f values calculated. Measurements were made from markings of the leading edge of the spots. The samples were identified by comparison of their R_f values to those of the standards. Development of 200 mm. was also tried on a 200 × 230 mm. plate using a larger developing tank.

Method for Fluorescent Plates (B).—The application of standards and samples, development, and air drying were identical to the procedure described for the neutral plates. Instead of the spray reagents, a short wave U.V. lamp (257 mμ) was used to observe the separation of the components, and the dark nonfluorescent spots were then marked. A summary of R_f ranges for the various sulfonamides tested is shown in Table II.

Figures 1 and 2 show typical, developed neutral chromatoplates of commercial products and standards using 1.2 and 1.5% water in the solvent system.

TABLE II.—APPROXIMATE R_f VALUES AND RANGES OBTAINED FOR VARIOUS SULFONAMIDES[a]

Sulfonamide	Av. R_f	Std. Deviation	Exptl. Ranges
Sulfacetamide	0.42	0.03	0.36–0.47
Sulfadiazine	0.47	0.03	0.43–0.53
Sulfathiazole	0.50	0.03	0.47–0.60
Sulfanilamide	0.53	0.02	0.48–0.57
Sulfamerazine	0.57	0.03	0.53–0.64
Sulfamethazine	0.64	0.04	0.59–0.72
Sulfaquinoxaline	0.66	0.05	0.62–0.75
Sodium sulfabromomethazine	0.77	0.04	0.70–0.83

[a] Data accumulated from 12 or more plates, using 1.5% water in the solvent system. In 1.2% water, the values for all except sulfacetamide, sulfathiazole, and sulfanilamide are reduced by at least 0.05.

RESULTS AND DISCUSSION

Procedure A.—Using the proper concentration of water, the sulfonamides, except VII, were sufficiently separated to allow identification by observation of the developed spot. Though the R_f value for VII closely approximated that for III, it was readily differentiated from the other by its yellow diazotization product. The identification of IV, if overlapped, was also possible due to its yellow diazotization product. Close observation of freshly sprayed spots showed IV to have a brown tint, V a red tint, and the others to be reddish-purple. On standing the colors faded.

The development of 200 mm. gave more distinct separation but did not justify the increased development time. Table III shows the various types of pharmaceutical combinations tested. The other components present did not interfere with the procedure.

Procedure B.—Though the total time consumed was reduced by the elimination of the spraying procedure, the sensitivity was also reduced. This was overcome by increasing the amount of sample applied to the plate. To increase the visibility of the samples, the diazotization and coupling can be carried out on the fluorescent plate. The reddish spots are then clearly visible on the yellow background.

Experimental plates were developed in solvent

TABLE III.—COMPONENTS OF PHARMACEUTICAL COMBINATIONS TESTED

Samples[a]	Sulfonamides I	II	III	IV	V	Others[b]
A and B	x	x	x			
C	x	x	x			Pink dye
D	x	x	x			Penicillin
E-G	x	x	x			
H	x	x		x		Green dye
I	x	x		x		
J	x	x			x	Yellow dye
K	x	x			x	Hyoscyamus
L and M	x	x				
N-P	x	x	x			
R-T	x	x	x			
U and W	x	x		x		
X	x	x			x	Sulfanilamide[c]
Z	x	x	x		x	Sulfanilamide[c]

[a] A to K, inclusive, represent tablets; L to Z represent suspensions. [b] Suspensions contained preservatives and suspending and flavoring agents. [c] Not stated by the formula.

systems containing various amounts of water. The water concentration was critical in the separation of sulfathiazole and sulfanilamide from the others. When these two were not present, an optimum range existed whereby the spots were sufficiently separated to allow easy identification.

A water concentration of 1.2% was found to be optimum for the separation of sulfathiazole from sulfadiazine, and 1.5% water was found to be optimum for the separation of sulfanilamide from sulfamerazine. Higher concentrations (1.8%) caused an overlapping of sulfanilamide and sulfadiazine; lower concentrations (1.2%) caused sulfanilamide to overlap the sulfamerazine. Sulfacetamide showed the best separation at a water level between 1.5 and 1.8%, though no overlapping occurred at the other levels tested.

Greater concentrations of water gave further movements for certain compounds but decreased the separation areas. A weakening of the adsorptive property of the silica gel G on some of the compounds by the water caused the increased movements. An anhydrous solvent system gave less separation, overlapping, and more tailing.

For the preliminary investigation of an unknown mixture, it is recommended that duplicate plates be prepared. One plate should be developed in the

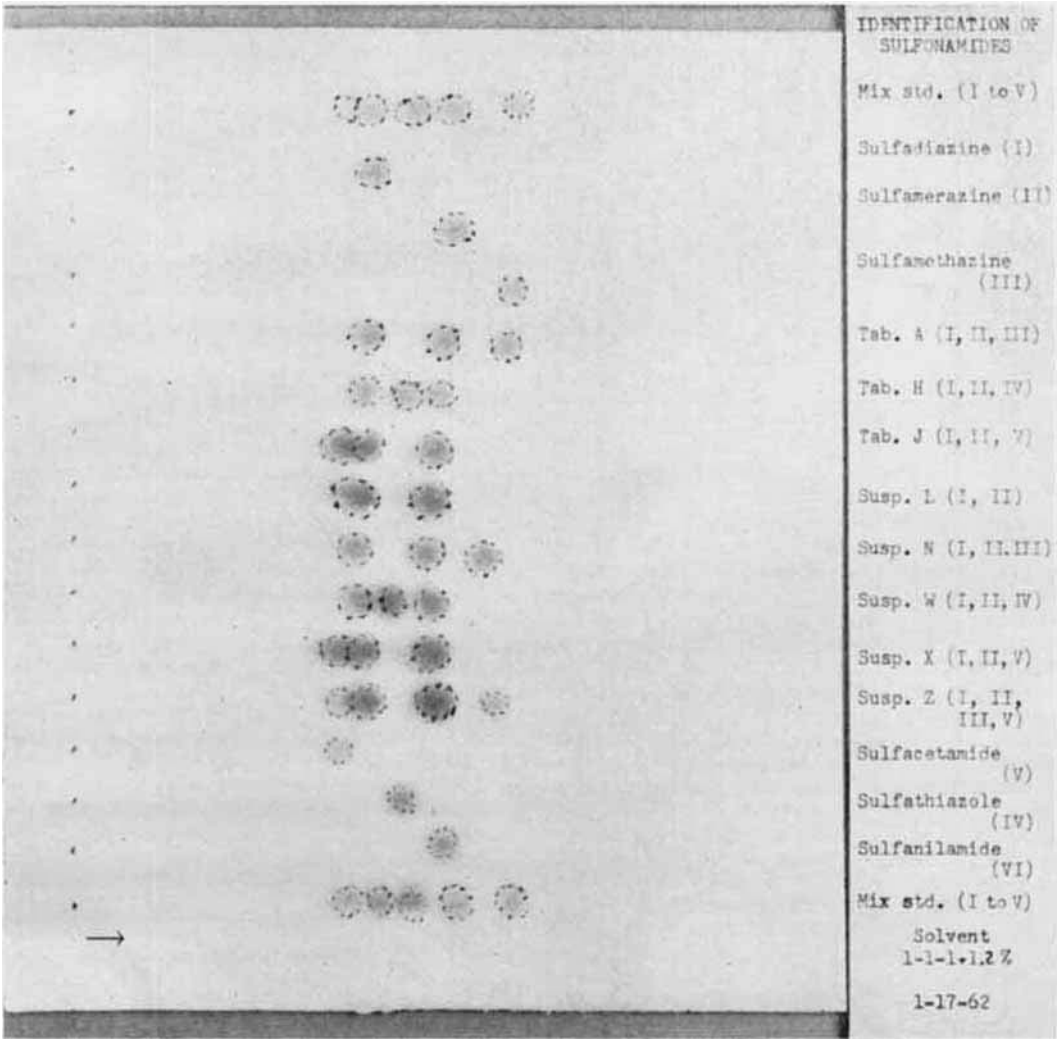


Fig. 1.—Thin-layer chromatographic separation of pharmaceutical combinations. Solvent: chloroform-heptane-ethanol (1:1:1 + 1.2% water).

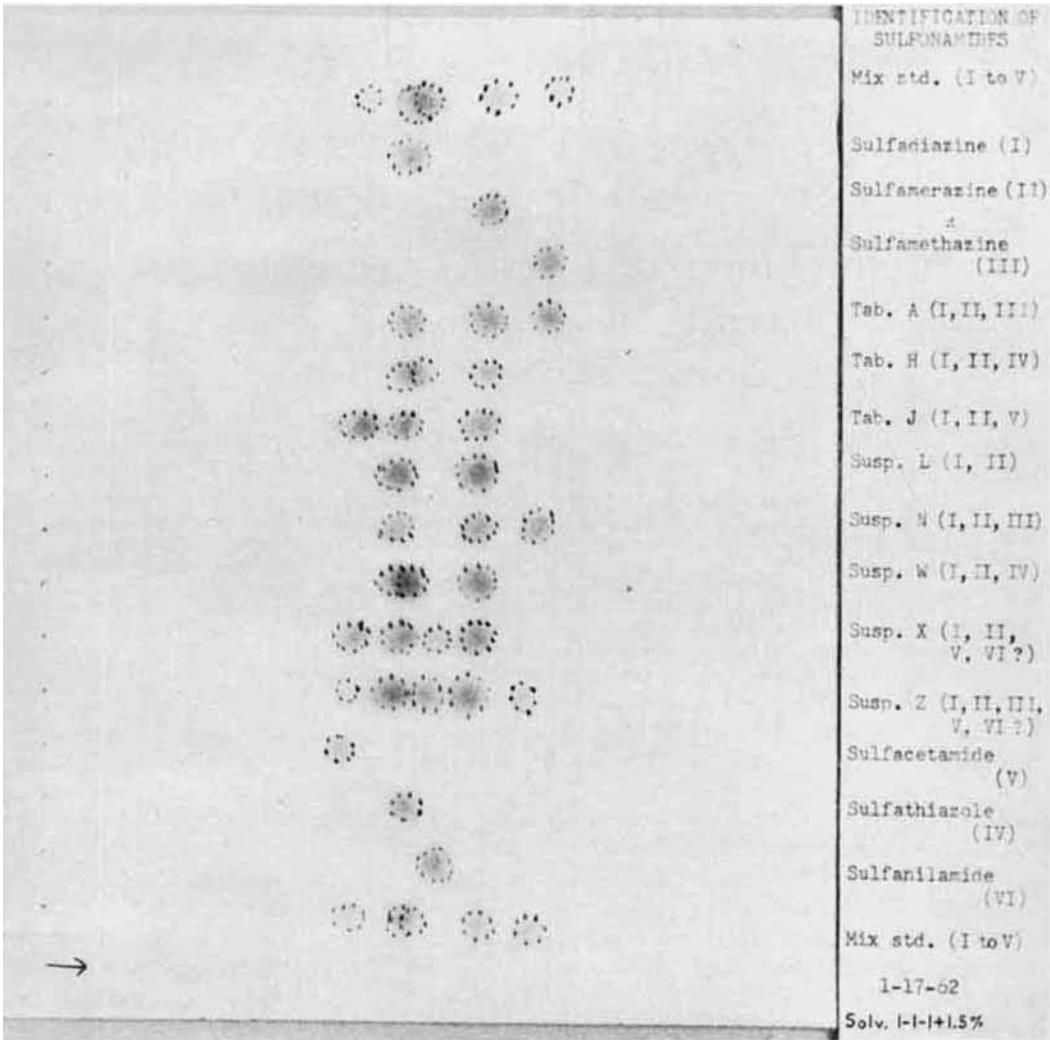


Fig. 2.—Thin-layer chromatographic separation of pharmaceutical combinations. Solvent: chloroform-heptane-ethanol (1:1:1 + 1.5% water).

solvent system containing 1.2% water and the other in the 1.5% water solvent system.

Ehrlich's reagent (*p*-dimethylaminobenzaldehyde, 1% in acidified ethanol) was tried as a replacement for the Bratton-Marshall reagent. Though it was less time consuming, the differentiation of VIII from III and the presence of IV, if overlapped, were not possible. Also, the outlines of the individual spots were not as distinct as those produced by using the Bratton-Marshall reagent.

Due to the uncertainty of reproducing R_f values from one plate to another, it is important to use standards along with the samples.

SUMMARY

1. A thin-layer chromatographic procedure for the identification of various sulfonamides individually and in combination has been developed.

2. Successful separation of mixtures containing the following sulfonamides has been obtained: sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, sulfanilamide, sulfaquinoxaline, and sodium sulfabromomethazine.

3. The identification of the following has been made when present in pharmaceutical combinations: sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, and sulfanilamide.

4. The concentration of water is critical for obtaining satisfactory separations of sulfathiazole and sulfanilamide from the other sulfonamides tested.

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Composition of Gum Turpentines from Twenty-Two Species of Pines Grown in New Zealand

By ALLETTE L. WILLIAMS and M. H. BANNISTER†

Vapor-phase chromatography, following steam distillation, has provided an effective means of identifying many components of the gum turpentines of oleoresins from 22 *Pinus* species, with the probable exception of the sesquiterpenes and some trace constituents. With the improved injection instruments, it has been possible to analyze samples as small as 0.5 Gm., thus allowing the study of very low-yielding or small trees. This work confirms the specific differences previously reported for the major constituents, with two notable exceptions, *P. muricata* and *P. montezumae*. The results suggest the existence of two varieties of *P. muricata*, distinguishable by a wide difference in turpentine composition.

THE GENUS *Pinus*, as described by botanists, comprises about 100 species, several of which are divided into subspecies or varieties. The chemical composition of the gum turpentine (*i.e.*, the volatile fraction of the oleoresin obtained by wounding the sapwood) has been reported for 92 species and 2 varieties, as summarized by Mirov (1). This work, requiring oleoresin samples of the order of 500 Gm., was done in the main by fractional distillation of the turpentine, followed by determination of the densities, optical rotations, and refractive indexes of the fractions, and finally by the preparation of crystalline derivatives. It was limited by the inadequate methods for isolating and identifying many of the individual compounds which occur only in trace amounts. Physical methods such as vapor-phase chromatography and infrared spectroscopy have simplified the study of turpentine composition, and have the great advantage of being applicable to very small samples. With vapor-phase chromatography alone, however, the comparison of retention volumes depends on the availability of pure reference compounds. Purified samples of terpenes are not readily available and their preparation is often difficult and time-consuming, especially when the compounds occur only as trace quantities. Nevertheless, the value of vapor-phase chromatography in the terpene field has already been recognized and the technique has been used by many workers (1–6).

A satisfactory vapor-phase-chromatography technique for studying the turpentine composition of *P. radiata*, *P. attenuata*, and hybrids between these two species has been developed (7). While extending this work to study individual variation in *P. radiata* (8) we decided to try the technique on a number of other species of pines grown in New Zealand. This would not only test the efficiency of the method for analyzing more complex turpentines, but would also provide a comparison between species grown in New Zealand and elsewhere.

EXPERIMENTAL

Method of Sampling.—All the oleoresin samples except one were collected from single trees growing in or near the New Zealand Forest Service Arboretum at Whakarewarewa, near Rotorua, in February 1961. The remaining sample was collected from a single tree of *P. muricata* on Huckleberry Hill, near Monterey, Calif., in 1960. Holes, 2.5 cm. in diameter, were bored into the sapwood and close-fitting tubes were inserted. With this method the oleoresin had minimum contact with the outside air, and the time required to collect the sample was usually 1 or 2 days, and in no case more than 7 days. The oleoresin samples were tightly

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Accepted for publication March 5, 1962.
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TABLE I.—COMPOSITION OF TURPENTINES OF OLEORESINS FROM *Pinus* SPECIES[a]

Species	Yield of Turpentine, %	*n*-Heptane	Unidentified:	*n*-Octane:	*n*-Nonane:	α-Pinene:	Camphene:	β-Pinene:	β-Myrcene:	Δ³-Carene:	α-Phellandrene:	Limonene:	β-Phellandrene:	*p*-Cymene:	*n*-Undecane:	Unidentified:	References
Relative retention ratios		0.14	0.23	0.29	0.64	1.00	1.21	1.54	1.78	1.94	2.08	2.48	2.63	2.94	3.12	3.88	
P. leiophylla	26.4					93.0	1.5	3.5	1.0			*t*	1.0				13*a*
P. pinea	27.9					2.5		*t*	2.5			95.0					18
P. jeffreyi	n.d.	86.0	*t*	*t*	1.5	*t*		*t*	2.5		*t*	*t*	5.5		4.5		1
P. torreyana	24.2	2.0		*t*	2.0	2.5	*t*	*t*	3.5			81.0			8.5		1
P. coulteri	17.5	1.0			3.5	44.5	1.5	4.5	16.5			3.0	16.0		9.5		10
P. coulteri[b]	17.5	*t*			1.5	29.5		18.0		*t*			49.0				
P. sabiniana	14.2	99.0		*t*	1.0	*t*								*t*			1
P. massoniana	31.1					91.5	2.5	2.5	1.0			1.0	1.5				1
P. thunbergii	23.6					89.0	2.0	5.0	1.5			1.0	1.5	*t*			1
P. luchuensis	24.1					85.0	2.0	5.0	1.5			1.0	4.5	1.0			1
P. nigra	25.4					93.0	2.5	2.0	1.0			1.0	*t*				11
P. ponderosa	23.8				*t*	6.5	*t*	35.0	7.0	37.0		14.5	*t*	*t*			12–16, 22
P. palustris	18.2					65.5	1.0	31.0	1.0				1.0				1
P. elliotii	17.5					59.0	2.0	34.5	2.0			*t*	2.5				1
P. taeda	n.d.					87.5	2.0	3.5	3.5	*t*		3.5	*t*				17
P. montezumae	24.6					8.5	*t*	2.0	1.5	87.0		*t*	1.0	*t*			13*a*, 24, 25
P. halepensis	32.9					90.5	2.5	4.5	2.5	*t*		*t*	*t*				18
P. pinaster	24.1					90.0	1.0	6.5	1.5			1.0	*t*				1
P. rigida	17.8					35.5	*t*	42.5	2.0		*t*	11.0	9.0				13
P. banksiana[b]	n.d.					84.5	*t*	13.0		*t*			2.5				12
P. contorta[b]	n.d.					2.0	*t*	3.0		4.0			90.0				1
P. patula	n.d.					6.0	*t*	1.5	2.0	5.0	1.0	*t*	82.5			1.0	1
P. muricata (A)[b]	22.3					2.5	*t*	5.0		86.5		*t*	*t*				
P. muricata (B)	26.4					13.0	*t*	3.5	2.0	80.5		*t*	*t*	1.0		*t*	
P. muricata (C)	23.2					6.0		3.5	2.5	86.5		*t*	*t*	1.5			
P. muricata (D)	25.2					2.5		3.5	2.5	90.0		*t*	*t*	1.5			
P. muricata (E)	27.9					97.0	1.5	1.5	*t*			*t*	*t*				26

[a] Values expressed as weight per cent; *t* indicates < 1%. [b] Samples analyzed directly after distillation at 120° and helium flow rate 21 ml./min.

stoppered and stored at about 0° whenever possible until required for analysis.

Analysis.—The oleoresin samples (20 Gm. or less) were steam-distilled at atmospheric pressure and the distillates (400 ml.) collected in ice-cooled flasks. The distillation time was about 40 minutes. The yields of the steam-volatile fractions, except from the very small samples, are given in Table I. When steam distilling the smaller oleoresin samples (*i.e.*, less than 2 Gm.) the distillate was collected in a narrow-bore syphoning tube, designed so that the steam-volatiles could be sampled directly. The turpentine samples were stored at 0° between analyses.

The steam-volatiles were chromatographed on a Perkin-Elmer vapor fractometer, model 154C, in conjunction with a Leeds and Northrup Speedomax type G recorder. Columns 1 M. in length and 4–5 mm. in diameter were packed with the following materials: 20% di-isodecylphthalate on Celite 545 (Johns-Manville); 23% squalane (May and Baker) on Celite 545; 11% squalane (May and Baker) on Celite 545; 20% silicone oil DC-200 on Celite 545; 18% polyethylene glycol (Carbowax 1500) on Chromosorb R (60–80 mesh); and 15% silicone oil (May and Baker) on Chromosorb R. The columns were tested at temperatures from 80 to 120° and with various helium flow rates. The most satisfactory overall separation of the constituents of the turpentines was obtained with a column of di-isodecylphthalate on Celite 545 at a temperature of 80° and a helium flow rate of 116 ml./min. The majority of the samples were analyzed under these conditions.

A Hamilton microliter syringe (0–10 μl.) was used to inject most samples into the gas stream. For the remaining oleoresin samples (*i.e.*, 0.5–1 Gm.) which produced only trace amounts of turpentine, a Perkin-Elmer microdipper (1 μl.) was used. The volume of steam-volatiles analyzed was varied from 0.4 to 1 μl., depending on the number of constituents present and their proportions. Each analysis was run in duplicate and the mean values reported to the nearest 0.5%. The areas under the peaks were determined with a planimeter, as some peaks were incompletely resolved, and areas of less than 1% of the sum of the peak areas were quoted as trace quantities.

Relative retention ratios using α-pinene as the reference were determined for authentic samples of known turpentine constituents with the di-isodecylphthalate/Celite column at 80°, and also with the silicone oil DC-200/Celite column at 80° as a check. The identities of the individual components of the turpentines being investigated were established by comparison with these ratios, which are given in Table II. Two minor constituents present in the turpentines have not been identified.

The turpentines were initially analyzed immediately after distillation on a di-isodecylphthalate/Celite column (1 M.) at 120° with flow rate of 21 ml./min. Limonene, β-phellandrene, *p*-cymene, and *n*-undecane peaks were not distinguishable at this temperature but were more satisfactorily resolved at a lower temperature of 80°. β-Pinene and β-myrcene were also separated at this temperature. The analyses were therefore repeated at a temperature of 80° and a flow rate of 116 ml./min. approximately 2 months after steam distillation. The compositions (weight percentages) of the steam-volatiles determined under these conditions are listed in Table I. As the analyses of *P. banksiana*, *P. contorta*, and *P. muricata* (A) could not be repeated at 80° owing to insufficient samples, the compositions of these species determined at 120° are reported. In the case of *P. coulteri*, the compositions found at both 120 and 80° are given.

Typical chromatograms, shown in Figs. 1, 2, and 3, were obtained under the following conditions: length of column, 1 M.; internal diameter of column, 4 mm.; column packing, 20% di-isodecylphthalate on Celite 545; temperature, 80°; helium flow rate, 116 ml./min.; recorder range, 8.

DISCUSSION

In this study it has been assumed that the mole responses of the constituents to the thermal conductivity cell detector are identical and, therefore, that the area under each peak is proportional to the mole per cent of that component. Jamieson (9), however, has shown that this assumption does not hold strictly when nitrogen is used as the carrier gas, and it is probable that small differences in the mole responses of the terpenes would also be found with helium. In a further investigation we propose to purify samples of the various constituents by preparative-scale gas chromatography and to determine their mole responses using helium as the carrier gas. It is expected that the necessary correction factors will be quite small. The purified terpenes will also be used to obtain optical rotation data.

We have also assumed that isomerization, polymerization, or decomposition of the turpentine constituents did not occur on the column di-isodecylphthalate on Celite 545. The catalytic effects of the supporting materials on terpenes have been discussed by many workers and Celite has been considered to be fairly satisfactory (2, 4, 5). However, Matsuura, *et. al.* (6), preferred crushed crystallized quartz which had been previously treated with nitric acid. In the present work, samples α- and β-pinenes chromatographed on a column of silicone oil (May and Baker) on Chromosorb R gave more than one peak, suggesting that the supporting medium or liquid phase was suspect. No

Table II.—Relative Retention Ratios for Terpene Hydrocarbons and Related Compounds at 80°

Compound	Relative Retention Ratios: Di-isodecylphthalate	Relative Retention Ratios: Silicone oil DC-200
n-Heptane	0.14	0.18
n-Octane	0.29	0.38
n-Nonane	0.64	0.79
α-Pinene	1.00	1.00
Camphene	1.21	1.07
β-Pinene	1.54	1.33
β-Myrcene	1.78	1.42
Δ^3-Carene	1.94	1.64
α-Phellandrene	2.08	1.56
Limonene	2.48	1.87
β-Phellandrene	2.63	1.85
p-Cymene	2.94	1.74
n-Undecane	3.12	3.40

such effect was detected when the pure compounds were chromatographed on a silicone oil DC 200/ Celite 545 column.

Although it has already been shown in the hybridism studies involving *P. radiata* and *P. attenuata* (8) that the ratio of the α-pinene to β-pinene content is not affected by ultraviolet light, storage time, or by distillation, this has not been established for the other terpenes encountered in these investigations. No doubt the paraffin hydrocarbons are unaffected. α- and β-Phellandrenes are known to be rather unstable compounds and to polymerize readily. This was demonstrated by the marked decrease in the β-phellandrene content of *P. coulteri* observed when the analysis of the turpentine was repeated at 80° after storage at 0° in the dark for 2 months. Hence, the composition of the volatile fraction of *P. coulteri* determined at 80° and without allowance for the polymerized phellandrene should be disregarded. Although the β-phellandrene content of *P. patula* was not affected during the same storage period, a similar decrease in the β-phellandrene content was subsequently shown. Polymerization of β-phellandrene probably also occurred during steam distillation, as suggested by Mirov (10) in his study of *P. coulteri*. He showed that polymerization was greatly decreased by distilling the turpentine under reduced pressure.

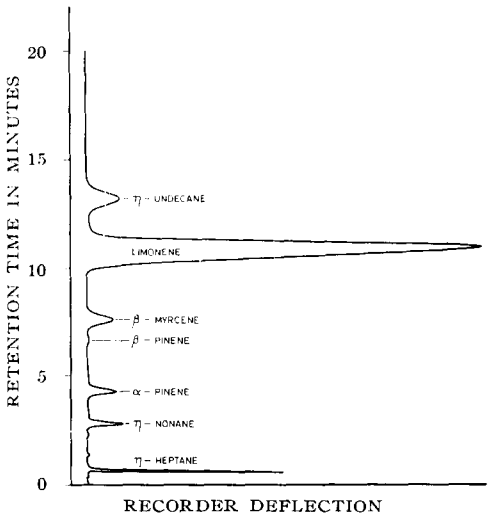


Fig. 1.—Chromatogram of turpentine from *P. torreyana*.

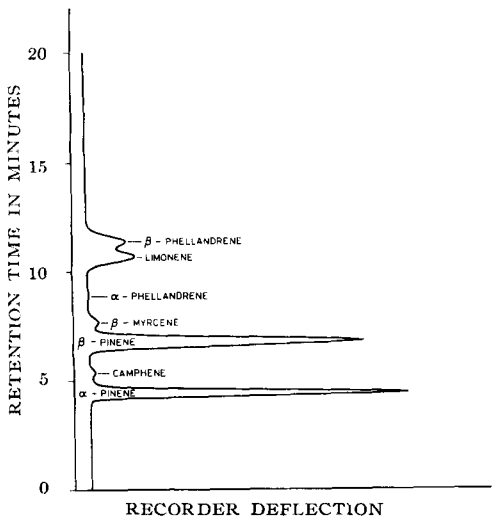


Fig. 2.—Chromatogram of turpentine from *P. rigida*.

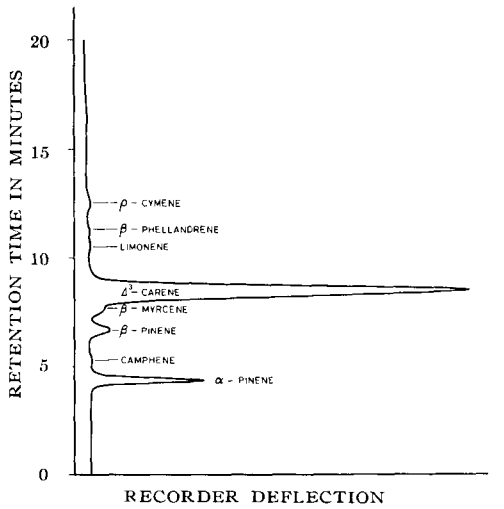


Fig. 3.—Chromatogram of turpentine from *P. muricata* (B).

The possibility of changes in the turpentine composition during collection of the oleoresin sample cannot be overlooked. Although precautions were taken during the sampling operation, these were probably not entirely adequate in preventing some changes in the less stable turpentine constituents.

An unidentified compound in concentration from 1 to 7% (relative retention ratio 3.00 at 120°) was detected in the turpentines of *P. coulteri*, *P. ponderosa*, *P. contorta*, *P. patula*, *P. montezumae*, and *P. muricata* (samples A, B, C, D) when chromatographed immediately after distillation at 120°. This compound was apparently degraded on standing.

In view of these considerations the compositions of the turpentines given in Table I cannot be assumed to be identical with those in the living trees.

A comparison of the results given in Table I with those already published suggests that high-boiling constituents, such as the sesquiterpenes, were not detected. While we have reduced the possibility of changes in the turpentine caused through prolonged steam distillation, it is probable that the high-boiling constituents have remained in the rosin fraction of the oleoresin. Distillation of the oleoresin under reduced pressure is necessary to isolate all the volatile components (1). On the other hand, it is possible that in chromatographing the turpentines at the low temperature of 80°, the high-boiling constituents have remained on the column.

β-Myrcene appears to be more widely distributed in the genus *Pinus* than was hitherto thought. Mirov (1) notes that its presence has been recorded in eight species including *P. nigra* (11), *P. ponderosa* (12–16), *P. taeda* (17), and *P. halepensis* (18). While *P. coulteri* was found to contain 18% β-pinene when chromatographed at 120° (Table I), the analysis of this sample at 80°, even though it is not entirely reliable, suggests that probably the greater proportion of this 18% would have been myrcene. The ease of polymerization of β-myrcene noted by McGimpsey and Murray (19) in the study of *Podocarpus spicatus* (matai) could perhaps account for the fact that myrcene has seldom been found in pines.

The occurrence of β-phellandrene and camphene in small amounts is also more widespread than was supposed (1). α-Pinene, not detected previously in *P. pinea* and *P. torreyana*, has been found in the New Zealand specimens of these species.

We have tentatively identified *p*-cymene in *P. sabiniana*, *P. luchuensis*, *P. ponderosa*, *P. montezumae*, *P. thunbergii*, and *P. muricata* (samples B, C, D). This compound has previously been reported only in *P. sylvestris* (20) and *P. taeda* (11). α-Phellandrene has also been tentatively identified in *P. patula*, *P. rigida*, and *P. jeffreyi*.

Two or more of the paraffin hydrocarbons *n*-octane, *n*-nonane, and *n*-undecane have been tentatively identified in each of the turpentines containing *n*-heptane, *P. jeffreyi*, *P. torreyana*, *P. coulteri*, and *P. sabiniana*.

Refinements in the experimental procedure such as the use of capillary columns, the extremely sensitive ionization detectors, temperature programming, or preliminary separation and concentration of fractions of the turpentines, could establish the existence of further components.

Botanical Aspects

In comparing the results with those summarized by Mirov (1) we find, in general, a close agreement, at least for the major constituents. This was to be expected, because Mirov (1, 21) has found that there is little variation throughout the growing season within the individual tree, and that apparently the composition of the turpentine is affected little, if at all, by variations in the habitat. He has concluded that composition of turpentine is a stable, "genetically fixed character."

On the other hand, it was also to be expected that our results would differ, in some ways, from those already published. Some differences may have resulted from variation in the methods of collection and analysis. Others may have arisen from genetic variation between groups of trees, or between individual trees, within a species. Little is known about variation of turpentine composition within species, but the evidence (1) suggests that some species are nearly uniform (*e.g.*, *P. merkusii*), whereas others are very heterogeneous (*e.g.*, *P. ponderosa*). There is, therefore, quite a high probability that our results, most of which represent only one individual tree per species, would include some which deviate markedly from the values reported previously. In particular, we wish to discuss three species which evidently vary widely in turpentine composition.

P. ponderosa.—This species has a wide geographic distribution, and has become genetically differentiated into a number of morphological and physiological forms. Analyses by Mirov and coworkers (12–16) of turpentines from 12 widely separate localities within its natural range, and another analysis by Goldblatt and Burgdahl (22) have revealed consistently high percentages of Δ^3-carene and, generally, of β-pinene. But there is a wide variation, both in the proportions of the major constituents and in the occurrence and percentages of the minor constituents. Our result falls well within this range of variation, and, in so far as it is comparable with Mirov's data, it suggests a close kinship between the New Zealand tree and the *P. ponderosa* of southwest Idaho or California.

P. montezumae.—This name is applied to a complex assortment of populations, most of which are found in Mexico. Martinez (23) has formally divided the complex into two varieties and one form. Two independent analyses by Iriarte (24) and Mirov (13*a*) of samples collected near Uruapan, Michoacan, agreed in finding α-pinene to constitute 96–98% of the turpentine. For a sample from Chiapas, Iloff and Mirov (25) reported the composition: 72% α-pinene, 7% limonene, 8% heptane, and 8% longifolene. Mirov (1) has concluded that these results may represent two distinct varieties, or two distinct species, or possibly one species in Michoacan and a swarm of interspecific hybrids in Chiapas.

Our results might suggest that the New Zealand sample came from *P. ponderosa* rather than from *P. montezumae*, but this seems unlikely. In its morphological characters, the tree in question is much more like *P. pseudostrobus* than *P. ponderosa*, and it might well be *P. montezumae* var. *lindleyi* Loudon. Further work on the turpentine composition of *P. montezumae* and related species is needed.

P. muricata.—Our sample A of this species contained about 86% of Δ^3-carene (Table I). It was collected on Huckleberry Hill, near Monterey, Calif. Mirov (26) had previously analyzed a sample collected near Fort Bragg, about 350 km. north of Monterey, and found the turpentine to consist almost entirely of α-pinene (98–99%). Some unpublished work by Mirov indicated that the turpentine of the Fort Bragg population might differ considerably in its optical rotation from that of the Huckleberry Hill population (27). Our result for the Huckleberry Hill sample was open to various interpretations, and as a check we sampled four trees growing near Rotorua.

Three of these belonged to a group which, from their circumstances and morphological characters, were judged to be very closely related to one another. The uniformity of their turpentines supports this judgment (Table I, samples B, C, and D). The fourth tree (sample E) was an isolated specimen, some 8 km. away. It differed considerably in morphological character from the others and yielded turpentine essentially the same as that of the Fort Bragg trees sampled by Mirov (26). Although only superficial observations have been made on the morphology of these four trees and of *P. muricata* elsewhere in New Zealand, we suggest that the data may represent a taxonomic discontinuity in *P. muricata*, two groups being distinguishable as follows: (*a*) somewhat squat, bushy trees with hemispherical crowns when grown in the open; dense green foliage; extremely spiny cones; and turpen-

tine predominantly of Δ^3-carene; (*b*) generally taller trees with conical crowns; foliage distinctly bluish, the needles thicker than in (*a*); cones with the apophyses more rounded on the outer side and generally less spiny than in (*a*); and turpentine predominantly α-pinene.

Unfortunately, the lineage of the New Zealand specimens cannot be traced back to a known provenance in California. Nevertheless, the scheme suggested not only fits the chemical facts so far, but also agrees closely with the morphological work of Duffield (27). His conclusion was that the mainland populations of *P. muricata* could well be divided into two varieties, one of which, including the Huckleberry Hill population and the typical form near San Luis Obispo, stretched from the Annapolis area southward, while the other, including the Fort Bragg population, stretched from Annapolis northward.

Further close collaboration between botanists and chemists studying this species is highly desirable; particularly useful would be a detailed population analysis in the Annapolis area where, according to Duffield (27), the trees show a morphological transition between the typical variety and the northern one.

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By SHU-SING CHENG†, SIGURDUR JONSSON‡, and FRED T. SEMENIUK

α- and β-Amino ketone analogs of amino acids were tested *in vitro* against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus anthracis, Saccharomyces cerevisiae,* and *Candida* spp. by serial broth dilution and agar diffusion methods and found to be active as growth-inhibitors at concentration levels which varied with the test culture. The study of the growth curve of a test bacterium in a liquid medium with and without the presence of the drug provides an accurate means for the *in vitro* evaluation of the antibacterial activity of potential chemotherapeutic agents. It serves also as a means for studying the dynamics as well as the mode of antibacterial action. Since the generation time and the numbers of generations of microbial growth occur before stationary phase can be calculated, the inhibitory activity of different drugs may be compared on this basis.

THE growth-inhibitory activity of β-amino ketone analogs of β-alanine against *Escherichia coli* and *Staphylococcus aureus* has been reported recently (1). This leads to speculation that α-amino ketone analogs of α-amino acids might possess similar activity. This study was then undertaken in order to investigate the potential antimicrobial action of these two categories of amino ketone analogs of amino acids.

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Presented to the Scientific Section, A.PH.A., Chicago meeting, April 1961.
Abstracted in part from a thesis submitted by Shu-Sing Cheng to the Graduate School of the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Recipient of 1961 Lunsford Richardson Pharmacy Award. Present address: Department of Chemistry, Clark University, Worcester, Mass.
‡ Deceased.

PROCEDURES AND RESULTS

There are three general methods for determining drug sensitivity of bacteria currently in use: the serial broth dilution, agar diffusion, and agar plate dilution methods. For the *in vitro* evaluation of antimicrobial activity of amino ketone hydrochlorides, the first two methods were selected.

Agar Diffusion Method.—*Staphylococcus aureus*, *Bacillus anthracis*, and *Pseudomonas aeruginosa* were used as test organisms. Stock culture was maintained on slants of peptone-casein agar and transferred to fresh slants about once a week. The assay plates and inocula were prepared according

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PROCEDURES AND RESULTS

There are three general methods for determining drug sensitivity of bacteria currently in use: the serial broth dilution, agar diffusion, and agar plate dilution methods. For the *in vitro* evaluation of antimicrobial activity of amino ketone hydrochlorides, the first two methods were selected.

Agar Diffusion Method.—*Staphylococcus aureus*, *Bacillus anthracis*, and *Pseudomonas aeruginosa* were used as test organisms. Stock culture was maintained on slants of peptone-casein agar and transferred to fresh slants about once a week. The assay plates and inocula were prepared according

TABLE I.—ANTIMICROBIAL ACTIVITIES OF AMINO KETONE HYDROCHLORIDES TESTED BY AGAR DIFFUSION METHOD

Hydrochloride[a]	Diameter, mm., of Inhibitory Zone against Microorganism Exhibited by the Indicated Quantity of Drug			
	Bacillus anthracis		*Pseudomonas aeruginosa*	
	1 mg.	2 mg.	1 mg.	3 mg.
n-Hexyl β-aminoethyl ketone	9	11	..	..
n-Heptyl β-aminoethyl ketone	13	32	..	..
Phenyl β-aminoethyl ketone	0	0	0	16
p-Tolyl β-aminoethyl ketone	0	0	0	10
2,5-Dimethylphenyl β-aminoethyl ketone	8	11	0	11
Phenyl aminomethyl ketone	10	16	..	..
o-Tolyl aminomethyl ketone	0	13	0	11
p-Tolyl aminomethyl ketone	7	16	..	..
3,4-Dimethylphenyl aminomethyl ketone	9	17	0	14
p-Tolyl α-aminoethyl ketone	0	7	0	10
3,4-Dimethylphenyl α-aminoethyl ketone	0	8	..	..
4-Phenyl-4-keto-2-amino-butyric acid-(1)	8	11	..	..
2-Amino-1-indanone	16	22	10	18

[a] See references (1) and (7) for the preparation of these compounds.

to U.S.P. XV method for penicillin microbial assay (2). The medicated disk method was used instead of cylinder-plate method. Peptone agar and peptone-casein agar were used for preparing the supporting agar layer and seeding layer, respectively.

The medicated paper disks were gently placed on the surface of the inoculated agar plate. For reference, sulfanilamide (5 mg.), sodium sulfamerazine (3 mg.), and sodium sulfapyridine (3 mg.) were included. In the case of sulfanilamide, the drug was placed directly on the surface of the inoculated agar plate without using the paper disk. All the plates were incubated at 37° for 16 to 18 hours, after which the growth responses were examined and the diameters of the clear zones of growth inhibition were measured on a bacterial colony counter. The results are summarized in Table I. 2-Amino-1-indanone hydrochloride exhibited a rose-red inhibitory zone on the surface of agar plate inoculated with *B. anthracis*, whereas phenyl aminomethyl ketone and *p*-tolyl aminomethyl ketone hydrochlorides each exhibited a rose-red inhibitory zone with a surrounding zone of growth-stimulation when tested under the same conditions. *o*-Tolyl aminomethyl ketone and 3,4-dimethylphenyl aminomethyl ketone hydrochlorides each showed yellow to brownish inhibitory zone surrounded by a zone of growth-stimulation when tested against *B. anthracis* and *P. aeruginosa*.

Of the 17 compounds tested, 2-amino-1-indanone and *n*-heptyl β-aminoethyl ketone hydrochlorides (2 mg. each) exhibited the widest zones of inhibition against the growth of *B. anthracis*. Sulfanilamide (5 mg.) produced very slight or no inhibition on the test organisms. Sodium sulfapyridine (3 mg.) and sodium sulfamerazine (3 mg.) were inactive.

Serial Broth Dilution Method.—*Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 P, *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida krusei* were used as test organisms. Stock cultures of *E. coli* and *Staphylococcus aureus* were maintained on trypticase-soy agar slant, while the stock cultures of yeast and fungi were maintained on Sabouraud dextrose agar slants. The culture media used in the test were a synthetic liquid medium (3) for *E. coli;* nutrient broth for *Staphylococcus aureus;* and Sabouraud liquid medium for *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida krusei*.

The inocula were prepared from 18- to 24-hr. bacterial broth cultures or from 3-day fungus and yeast broth cultures. For broth cultures, the test organisms were transferred into the appropriate liquid medium and incubated at 37° for bacteria, and 30° for yeast and fungi. One drop (0.05 ml.) of 1:100 dilution of the broth culture in sterile distilled water was used to inoculate the medium. Viable cell counts were made from the serial dilutions of these inocula by the pour-plate method for bacteria, and by the surface-spreading method for yeast and fungi.

The stock solutions of amino ketone hydrochlorides were prepared by dissolving the drug in sterile distilled water to make a concentration of 5000 mcg./ml., and were sterilized by filtering through a Millipore filter.

For the conduct of the test, 10 15-ml. test tubes, each containing 5 ml. of the sterilized double-strength broth, were divided into duplicate series. A 1-ml. quantity of the stock solution of amino ketone hydrochloride was added to the first tube; 0.5 ml. of the same solution was added to the second; 0.5 ml. of a 1:2 and 1:4 dilution of the stock solution, respectively, was added to the third and fourth. The volume was made up to 10 ml. in each tube. The final concentration of the amino ketone hydrochloride in each tube was 500, 250, 125, 62.5, and 0 mcg. per ml., respectively. The last tube, without drug, served as organism growth control. Each tube was inoculated with 1 drop of a 1:100 dilution of the broth culture. Incubation was at 37° overnight for the bacteria, and at 30° for 3 days for yeast and fungi. The tubes were examined for growth inhibition of the organism at the end of each incubation period. The least amount of drug resulting in complete inhibition of growth was recorded as minimum inhibitory concentration. The results are summarized in Table II.

Mode of Antibacterial Action.—In order to investigate the mode of antibacterial action, a study of the growth curve of the test organism in a liquid medium, with and without the presence of drug, was then performed. The results are shown in Figs. 2 and 3 for the response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone and *n*-heptyl β-aminoethyl ketone hydrochlorides, respectively. The growth curve was based on viable cell counts obtained by the pour-plate technique.

Examination of the control growth curve Ia (the curve resulting from the growth of *Staphylococcus aureus* in the absence of drug) in Figs. 2 and 3 reveals that the curves follow the pattern of a typical growth curve of bacterium as shown in Fig. 1, except for the lack of a lag phase. This is explainable by the active physiological state of the bacterial cells (19-hr. culture).

In Fig. 2, curves IIa, IIIa, and IVa record the growth response of 14,600 cells of *Staphylococcus aureus* in nutrient broth in the presence of 125, 250, and 1000 mcg. of *o*-tolyl aminomethyl ketone hydrochloride per ml. of culture medium. Curves IIa and IIIa indicate that, at drug concentrations of 125 mcg. and 250 mcg./ml., 14,600 cells from the inoculum multiply by four to five generations to reach a final population of 3×10^5 cells, 8 hr. and 36 min. after inoculation and reach the stationary phase. The calculation involves solving the equation $b = a \times 2^n$, where b is the number of cells of the final population, a is the number of cells introduced into the medium from the inoculum, n is the number of generations by which the initial number of cells multiply to yield the final population (4). The calculation is shown as follows

$$b = a \times 2^n$$

$$n = \frac{\log_{10} b - \log_{10} a}{\log_{10} 2}$$

$$= \frac{\log_{10} 3 \times 10^5 - \log_{10} 14{,}600}{\log_{10} 2}$$

$$= 4.368 \text{ generations}$$

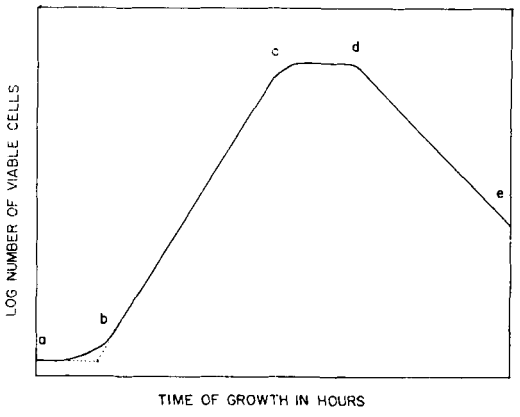


Fig. 1.—Typical growth curve of bacteria.

In contrast to curves IIa and IIIa, Ia indicates that, in the absence of drug, 14,600 cells continue the multiplication for 20.32 generations to yield a final population of 192×10^8 cells before the phase of decline is reached at 8 hr. and 36 min. after the inoculation was made

$$n = \frac{\log_{10} 192 \times 10^8 - \log_{10} 14{,}600}{\log_{10} 2}$$

$$= 20.32 \text{ generations}$$

On the other hand, as shown by curve IVa, representing a drug concentration of 1000 mcg./ml., 14,600 cells of *Staphylococcus aureus* did not increase in number, but decreased logarithmically even after brief contact with the drug. Eighty-eight minutes after inoculation and contact with the drug, the population dropped to 9000 cells, contrasting with the respective figures of 105×10^3, 213×10^3, and 696×10^3 cells in the presence of 250, 125, and 0 mcg. of *o*-tolyl aminomethyl ketone hydrochloride/ml. of culture medium, respectively. In the logarithmic phase, the curve IVa has a negative slope of −0.00239 in comparison with +0.00973, +0.01322, and +0.0197 for curves IIIa, IIa, and Ia, at respective drug concentrations of 250, 125,

TABLE II.—ANTIMICROBIAL ACTIVITIES OF AMINO KETONE HYDROCHLORIDES TESTED BY BROTH SERIAL DILUTION METHOD

Hydrochloride[a]	Minimum Inhibitory Concn. against Microorganism, mcg./ml. of Medium				
	E. coli	*Staph. aureus*	*C. albicans*	*C. krusei*	*Sacch. cer.*
n-Butyl β-aminoethyl ketone	500	...	...	...	...
n-Hexyl β-aminoethyl ketone	500	1000	2000	...	1000
n-Heptyl β-aminoethyl ketone	250	500	...	1000	...
Phenyl β-aminoethyl ketone	500	1500	...	...	...
p-Tolyl β-aminoethyl ketone	500	...	...	...	2000
2,5-Dimethylphenyl β-aminoethyl ketone	250	250	1000	2000	2000
Phenyl aminomethyl ketone	250	250	500	...	2000
o-Tolyl aminomethyl ketone	250	250	...	...	...
p-Tolyl aminomethyl ketone	250	250	...	...	...
3,4-Dimethylphenyl aminomethyl ketone	250	250	...	...	...
2-Furyl β-aminoethyl ketone	250	...	...	...	...
2-Thienyl β-aminoethyl ketone	125	...	...	...	...
p-Hydroxyphenyl α-aminoethyl ketone	125	...	...	...	...
p-Tolyl α-aminoethyl ketone	...	...	2000	...	2000
3,4-Dimethylphenyl α-aminoethyl ketone	125	200	...	...	...
Phenyl α-aminoethyl ketone	...	200	...	...	...
4-Phenyl-4-keto-2-amino-butyric acid	1000	...	...	...	...
2-Amino-1-indanone	125	63	500	250	...

[a] See references (1) and (7) for the preparation of these compounds.

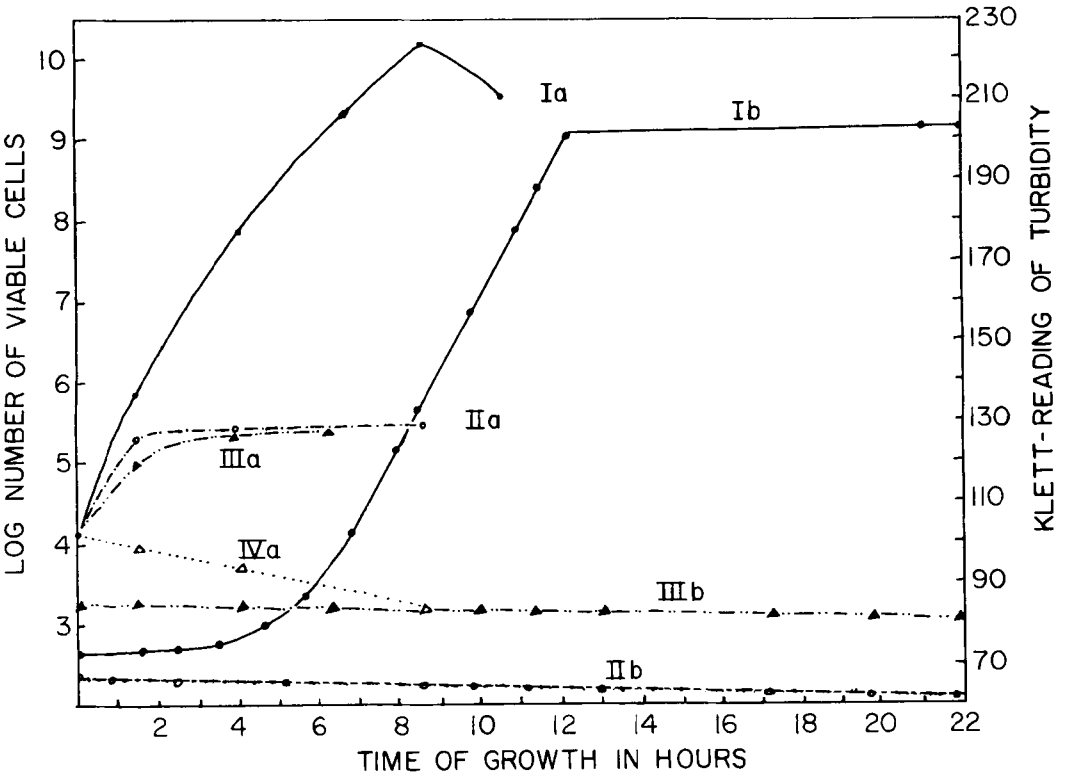


Fig. 2.—Growth response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone hydrochloride.

and 0 mcg./ml. of culture medium. The slopes of the straight lines represent the growth rate, R, during the logarithmic growth. They are simply calculated by solving the equation for logarithmic growth in terms of the variables of the growth curve

$$R = \frac{\log_{10} b_2 - \log_{10} b_1}{t_2 - t_1}$$

where t_2 and t_1 is the time at which the culture has viable cell count of b_2 and b_1, respectively. The data were taken from Table III at the end of 88 min. of incubation. The positive value of R represents the growth rate, whereas the negative value of R represents the death rate. From growth rate, the generation time, g, can be calculated by solving the following equation

$$b = a \times 2^n$$

$$\log_{10} b = \log_{10} a + n \log_{10} 2$$

$$\log_{10} b = \log_{10} a + \frac{t}{g} \log_{10} 2$$

$$\log_{10} b = \log_{10} a + \frac{0.301}{g} t$$

then

$$\text{slope} = R = \frac{0.301}{g}$$

and

$$g = \frac{0.301}{R}$$

TABLE III.—GROWTH RESPONSE OF *Staphylococcus aureus* TOWARD *o*-TOLYL AMINOMETHYL KETONE HYDROCHLORIDE IN NUTRIENT BROTH

Growth Time	Calculated Log No. Viable Cell/30 ml. —Drug Concentration mcg./ml. Medium— 0	125	250	1000
Zero (Inoculation)	4.1644	4.1644	4.1644	4.1644
1 hr. 28 min.	5.8426	5.3284	5.0212	3.9542
4 hr. 3 min.	7.9258	5.4771	5.4771	...
6 hr. 20 min.	8.9484	...	...	...
8 hr. 36 min.	10.2833	...	...	...
10 hr. 23 min.	9.4771	...	...	...

The averaged generation time calculated for cultures in the presence of *o*-tolylaminomethylketone hydrochloride at concentration of 0, 125, and 250 mcg./ml. is 15.78, 22.77, and 30.94 min., respectively. The elongation in generation time due to the presence of drug indicates the bacteriostatic effect of *o*-tolyl aminomethyl ketone hydrochloride.

Curve IVa reveals the bactericidal action of *o*-tolyl aminomethyl ketone hydrochloride at concentration of 1000 mcg./ml. The rate constant, k of the bactericidal action at this concentration is equal to 0.05726 min.$^{-1}$. This is calculated from the equation for logarithmic order of death (5)

$$k = \frac{2.303}{t} \log_{10} \frac{a}{b}$$

where a is the initial population and b is the final number of bacteria after contact with the drug for time period t. The death rate follows the rate of a

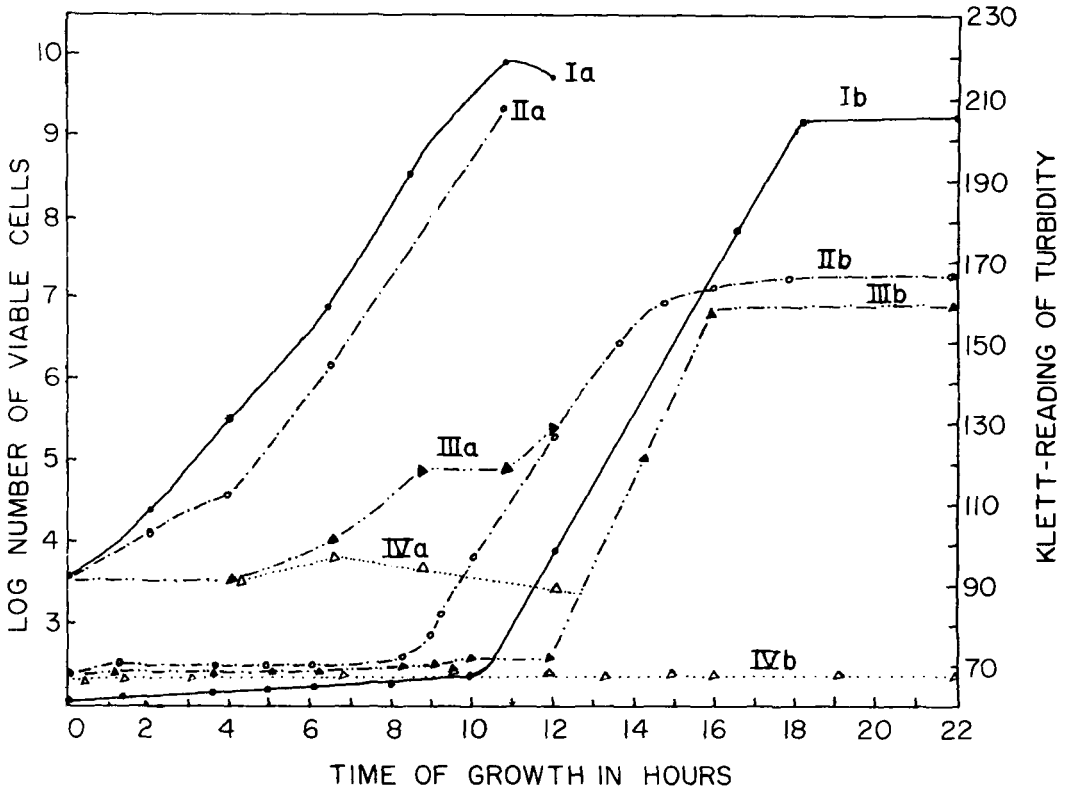


Fig. 3.—Growth response of *Staphylococcus aureus* toward *n*-heptyl β-aminoethyl ketone hydrochloride.

first-order reaction, which is usually observed for many bactericidals (6). It is still an open question whether this implies a simple inhibition of the rate of a single reaction sequence or is merely a coincidence.

From the mathematical treatment, it is obvious that *o*-tolyl aminomethyl ketone hydrochloride acts as a bacteriostat at low concentrations (*ca.* 250 mcg./ml.), and as a bactericide at higher concentrations (*ca.* 1000 mcg./ml.). This conclusion is true also for *n*-heptyl β-aminoethyl ketone hydrochloride, an amino ketone analog of β-alanine. The growth curves in Fig. 3 confirm this statement. In the above experiment, the growth responses were checked also by the turbidity reading of the culture. The turbidity curves are plotted in Figs. 2 and 3 and are designated with subscript *b*, in contrast to the use of subscript *a* for the viable population curve resulting from colony counting.

In order to investigate the mechanism by which β-aminoethyl ketone hydrochlorides act as antibacterial agents, an inhibition-reversal study with β-alanine and calcium L-pantothenate was performed. The results show that at inhibitor: metabolite molar ratio of 1.101:1 or 11.48:1, neither β-alanine nor calcium L-pantothenate possess any reversal activity in antagonizing the growth-inhibitory effect of *n*-heptyl β-aminoethyl ketone hydrochloride against *E. coli*.

DISCUSSION

The reversal of the growth-inhibitory effect of a compound by a structurally or biochemically related metabolite implies that the inhibitor in question prevents growth by interfering with a biochemical pathway involving that metabolite. The preliminary experiment on the inhibition-reversal study of *n*-heptyl β-aminoethyl ketone hydrochloride by either β-alanine or calcium pantothenate failed to demonstrate the antagonism at an inhibitor:metabolite molar ratio of 1.101:1 or 11.48:1 against *E. coli*. At a molar ratio of 0.805:1 glycine also failed to nullify the growth-inhibition of an amino ketone analog of glycine on a glycine dependent organism. These facts suggest that amino ketone analogs of amino acids may be valuable chemotherapeutic agents, and they may warrant further study.

In contrast to α-aminomethanesulfonic acid, the amino ketone analogs of glycine prepared for this work (7) are active in growth-inhibition against *E. coli* at concentration levels of *ca.* 1×10^{-3} mole. α-Aminomethanesulfonic acid has been found to be a glycine antagonist on test systems such as: the bacteriophage of *E. coli* (8), vaccina virus (9), and of different species of bacteria (10), but is not active against *E. coli* (11). This implies that amino ketones

TABLE IV.—GROWTH KINETICS OF *Staphylococcus aureus*[a] IN THE PRESENCE OF *o*-TOLYL AMINOMETHYL KETONE HYDROCHLORIDE

Drug Concn., mcg./ml.	Growth Rate, min.$^{-1}$	Generation Time, min.	Final Population, cell	Rate Constant of Bactericidal Action, min.$^{-1}$
0	+0.01907	15.78	696×10^3	...
125	+0.01322	22.77	213×10^3	...
250	+0.00973	30.94	105×10^3	...
1,000	−0.00239	...	9×10^3	0.05726

[a] Initial population, 14.6×10^3 cells of *Staphylococcus aureus* 6538P; culture medium, nutrient broth; incubation temperature, 37°. Data are average figures calculated for the first 88 min. of incubation.

may be more reliable than amino sulfonic acids as growth inhibitors when they are structurally related to natural amino acids. The observations that amino ketone analogs, $H_2NCH_2CH_2COR$, of β-alanine inhibit the growth of many microorganisms, whereas taurine, $H_2NCH_2CH_2SO_3H$, the amino sulfonic acid analog of β-alanine, functions as an essential metabolite in vertebrates and invertebrates have lent support to this viewpoint.

The study of the growth curve of *Staphylococcus aureus* in liquid medium, with and without the presence of drug, reveals the pattern of growth-inhibition. Two types of response to an inhibitor have been observed for *o*-tolyl aminomethyl ketone and *n*-heptyl β-aminoethyl ketone hydrochloride, respectively; these are referred to as types A and B. In type A, the plot of log viable cell count against time is linear up to 88 min. after inoculation, as shown by curves Ia, IIa, and IIIa in Fig. 2. This linear relationship is in agreement with the exponential growth law. The growth rate of a test bacterium is reduced by amino ketone hydrochloride; the degree of depression increases with increase in concentration of drug. Type A growth response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone hydrochloride corresponds to the type 1 response described by Moore and Boylen (12) for *E. coli* toward 5-bromouracil; while type B growth response of *Staphylococcus aureus* to *n*-heptyl β-aminoethyl ketone hydrochloride is equivalent to type 3 growth response of *E. coli* toward pyridine-3-sulfonic acid.

The study of the growth curves of test bacteria suggests an accurate method for evaluating the *in vitro* antibacterial activity of drugs with mathematical implication. It serves also, as a means for studying the kinetics as well as the mode of antibacterial action, and may be generally applicable in the screening of antimicrobial agents.

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Antienzymes to α- and β-Amylase

By J. J. STOCK and H. D. SANDERS†

Studies on the antigenicity of an α- and a β-amylase have been made. The use of incomplete Freund's adjuvant enhanced the amount of enzyme-neutralizing antibody formed by rabbits to active α-amylase (*Bacillus subtilis*), whereas, under similar conditions, the adjuvant did not enhance antienzyme formation to active β-amylase (barley). The use of this adjuvant prolonged the duration of antibody level produced by both of these enzymes. Acid-inactivated α-amylase was found to produce a circulating antibody in rabbits which will neutralize its homologous active enzyme. It is suggested that the determinant groups on this enzyme, which are responsible for antibody production, are not necessarily the same groupings responsible for activity on substrate.

COMPARATIVELY FEW observations of antibodies to α- and β-amylase have been reported. Sevag (1) has demonstrated that the antienzyme to malt amylase is specific in its enzyme-neutralizing capacity and will not neutralize the activity of an α-amylase. Furthermore, inactivated malt amylase was shown to be nonantigenic in the sense that sera from animals inoculated with this material possessed no antienzyme capacity. Recently, Nomura and Wada (2) have obtained rabbit antiserum against the amylase of *B. subtilis*. Also, Wada (3) has shown that the presence of heat-denatured Taka-α-amylase did not inhibit reaction between this active enzyme and its antibody.

No observations appear to have been made concerning the effect of an adjuvant on the antigenicity of these enzymes, or of any attempt to investigate the serologic relationship of an antienzyme to inactivated α-amylase. It was hoped that an investigation of this nature would help provide more knowledge concerning the molecular groupings of these enzymes which are responsible for activity on substrates and those which are responsible for antigenic activity, as well as the immunospecificity of these antigens when injected into rabbits over a comparatively long period of immunization. Previous investigators (4–9) have shown a diminution in specificity of an antiserum following a prolonged immunization period with various antigens.

MATERIALS AND METHODS

Antigenic Materials.—The α-amylase used in this investigation was obtained from a bacterial source (*Bacillus subtilis*) and the β-amylase was derived from barley; both of these materials were obtained commercially.[1]

Assay for Enzyme Activity.—Glycogen, $[\alpha]_D^{20} = +196$ to 197°, containing no reducing sugars, was the substrate used to demonstrate enzymic activity.[1] The assay method was that of Somogyi (10) for the determination of reducing sugars, as modified by Nelson (11) and Somogyi (12). All measurements for the concentration of reducing sugars, released from the substrate through enzymic activity at 37° for 15 minutes at a pH of 5.3, were made using a Bausch & Lomb Spectronic 20 at a wavelength of 510 mμ (13).

Serologic Tests.—A method for the indirect determination of antienzyme concentration through a modification of the Nelson-Somogyi procedure was performed as described elsewhere (13). The values reported for the reciprocal of serum dilutions represent the dilution of antibody, at the time of sampling recorded, which neutralized the enzymatic activity of the standard enzyme preparation used. It was found that naturally occurring amylase in rabbit sera at dilutions of 1:25 or higher would not affect assay results.

Adjuvant.—Incomplete paraffin-oil adjuvant, described by Freund (14), was prepared to contain 2 parts of paraffin oil (Marcol GX),[2] 1 part of Falba, and 2 parts of the desired concentration of enzyme. The incorporation of the amylases with the adjuvant base and the control tests have been reported elsewhere (13).

Sera.—All rabbits were bled by ear vein puncture, and the sera were preserved at −20°. No preservative was added to these sera.

Immunization.—Those rabbits which were immunized with the enzyme incorporated in the adjuvant base were injected subcutaneously in the scapular region over a period of 78 days. Each group of rabbits was immunized on the basis of equal amounts of total nitrogen. On this basis, 0.027 mg. nitrogen/mg. (± 0.002) of enzymic material promoted the same degree of hydrolysis of substrate, whether it was α- or β-amylase. Where amylase was used as an antigenic agent without the use of incomplete Freund's adjuvant, it was injected in the form of a sterile saline solution every 4 days, either intravenously or subcutaneously, depending on the experiment. The total amount of nitrogen injected was adjusted to be the same at the end of successive

Received December 11, 1961, from the Department of Bacteriology and Immunology and the Faculty of Pharmacy, University of British Columbia, Vancouver, B. C., Canada.
Accepted for publication January 17, 1962.
This investigation was aided by a grant from the President's Committee on Research, University of British Columbia, and from The Canadian Foundation for the Advancement of Pharmacy.

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[1] Nutritional Biochemicals Corp., Cleveland 28, Ohio.
[2] Supplied through the generosity of Imperial Oil Limited, Vancouver, B. C., Canada.

8-day periods for each group of rabbits during the first 44 days of the immunization period. After this time, further injections were made as shown in Figs. 1 and 2. Blood was collected from the animals at the intervals indicated in the graphs. All assays for antibody content were made at the conclusion of each immunization schedule.

Inactivation of α- and β-Amylase.—α-Amylase was inactivated according to a modification of the method used by Kneen, Sandstedt, and Hollenbeck (15) and as previously reported (13). Irreversible inactivation of this enzyme took place at a pH of 3.0 when held at 37° for 10 minutes in a 0.02 *M* biphthalate-hydrochloric acid buffer. According to previous findings (13), this method of acid inactivation could not be employed with β-amylase. Therefore, β-amylase in 0.02 *M* phosphate buffer of pH 7.2 was inactivated at a temperature of 55° after 30 minutes.

EXPERIMENTAL AND RESULTS

Route of Antigen Injection.—Using active β-amylase without adjuvant and on the basis of equal amounts of total nitrogen injected, the route of injection, whether it be subcutaneous or intravenous, does not appear to influence antienzyme formation in rabbits. These observations were made using a total of 11 animals.

Effect of Incomplete Freund's Adjuvant on the Antigenicity of α- and β-Amylase.—The rate of appearance of antibodies in the circulation was determined at various times during the immunization schedule and the findings are reported in Figs. 1 and 2. No enhancement in antienzyme formation to β-amylase was found using this adjuvant. Several rabbits from two distinct sources of albino New Zealand breeding stock of both sexes and weighing approximately 2.5 Kg. were used without success in attempts to obtain higher antienzyme titers. On the other hand a potentiation of approximately 10 times in antibody production was observed when α-amylase was used with the adjuvant. Antienzyme production to both antigens was maintained for a prolonged duration with the use of this adjuvant. (See Figs. 1 and 2 at the times of 54 and 100 days.)

Antigenicity of Active and Inactive Amylases.—As illustrated in Figs. 1 and 2, the α-amylase used is a comparatively good antigenic agent as compared to β-amylase derived from barley. β-Amylase inactivated by a mild heating, and using incomplete Freund's adjuvant does not produce detectable circulating antienzymes in rabbits. On the basis of total nitrogen injected, inactivated α-amylase has been shown to produce antienzymes in as high a titer as does the active form of the enzyme when incorporated into this adjuvant. This acid-inactivated α-amylase produced antienzymes which neutralized the activity of its homologous active enzyme on glycogen. Therefore, it appears that the determinant groups on this enzyme responsible for antibody production may not be the same groupings which are responsible for activity on substrate. Cross-neutralization tests with antienzymes to all antigenic agents used showed no apparent inhibition of activity on substrate, even though these antibodies had been produced after a comparatively intensive and prolonged schedule of immunization.

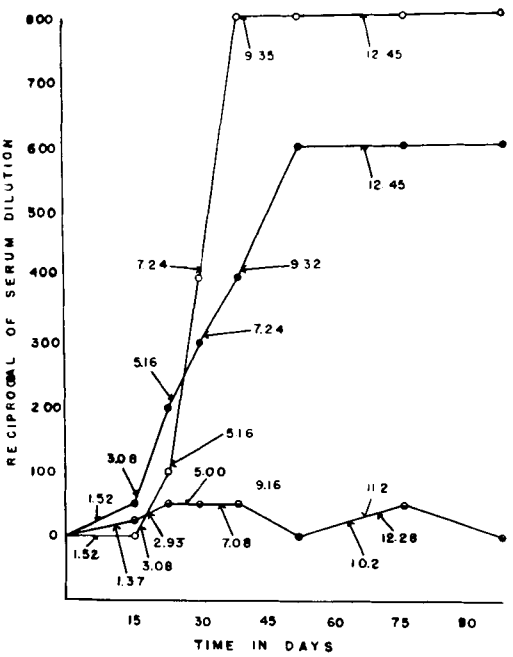


Fig. 1.—Inhibition of α-amylase by homologous antisera. The reciprocal of the antisera dilutions to α-amylase represents the median of the values obtained for each group of animals. Test bleedings were made just prior to injections as designated by points on the graphs. Numbers record cumulative amounts of total N injected at designated times. Despite the comparatively small number of animals used here, there was a consistent difference in the response of each group, and this was significant. O——O, Inactive α-amylase in adjuvant, 4 animals; ●——●, active α-amylase in adjuvant, 3 animals; ◒——◒, active α-amylase in saline, 2 animals.

DISCUSSION

The objective of this investigation was not a comparison of the antigenicity of α-amylase with that of β-amylase, as the origin of the materials used was from divergent sources. Also, we have made no assumption that these materials are to be considered as representative of this class of enzymes as our findings may not be similar to those which may be obtained with the use of other preparations. According to our findings, Sevag's observations concerning the immunospecificity of the antienzyme to β-amylase and its inability to neutralize the activity of an α-amylase, and the lack of antigenicity of inactivated β-amylase have been confirmed.

In this investigation we have shown that α-amylase from *Bacillus subtilis* is a better antigenic agent than β-amylase (barley) in the production of circulating antibodies in rabbits. Also, the enzymatically inactive form of this amylase is as good an antigen as the active α-amylase and will neutralize its activity.

Little and Caldwell (16, 17) have shown that the activity of α-amylase on a substrate requires that the primary amino groups be free, that tyrosine groups are not essential, and that sulfhydryl groups may be lacking entirely. Weill and Caldwell (18) have

reported that with β-amylase, primary amino groups are not essential for activity, whereas tyrosine and sulfhydryl groups are necessary. It may be surmised that these differences between the molecular structures of the two enzymes or the presence or absence of certain groups on the molecule of active β-amylase may help contribute to its comparatively poor antigenicity. According to our observations, the factors which lend themselves to acidic inactivation of α-amylase and its heat stability are not the same with β-amylase, as this enzyme is comparatively thermolabile (13).

Despite the fact that Little and Caldwell (16, 17) carried out their analyses on an α-amylase originating from pancreas, and the α-amylase used in this work was from a bacterial source, and that α-amylases from different sources may vary in characteristics such as stability, pH optima, etc., it is accepted generally that α-amylases do not differ with respect to their specific action on substrate, but only in their affinities for substrate (19).

McGeachin and Reynolds (20) have demonstrated that hog pancreatic and human salivary amylases are antigenic and that their respective antibodies have amylase-inhibiting properties. They have reported that the enzymatic site of these amylases is part of or located close to the antigenic site and is blocked or covered by the large antibody molecules. Using amylase of *B. subtilis*, Wada and Nomura (21) found that the neutralization reaction by antibody was inhibited by the presence of substrate or certain of its degradation products. They suggested that there may be a competitive relationship between substrate and antibody for combination at the same portion of the enzyme surface. Although the antigen-antibody interaction may mask enzymatically active sites, according to our observations, the activity site of α-amylase (*B. subtilis*) for substrate may be destroyed, but the antigenic site remains unaltered.

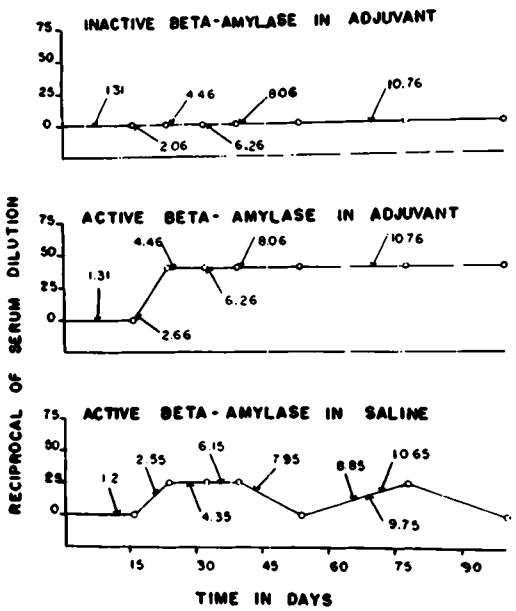


Fig. 2.—Inhibition of β-amylase by homologous antisera. The reciprocal of the dilutions of antisera to β-amylase represents the median of the values obtained for each group of animals. Test bleedings were made just prior to injections as designated by points on the graphs. Numbers record cumulative amounts of total N injected at designated times. Inactive β-amylase in adjuvant, 12 animals; active β-amylase in adjuvant, 7 animals; active β-amylase in saline, 11 animals.

In this work, we have shown that the inactivation of α-amylase by acidification does not decrease its antigenicity for rabbits, and according to Little and Caldwell's observations (16, 17), it appears that free primary amino groups on the α-amylase molecule acting as an antigen are not essential to its ability to produce antibody. To the authors' knowledge, the exact mechanism of acid inactivation for this molecule is not known, however, the addition of acid to a protein may alter its structure. Thus, the active site of an enzyme could be destroyed but its antigenic groups may remain unaltered. Since α-amylase activity is lost irreversibly by this method of inactivation, it is suggested that the amino groups are not free, and that this altered molecule either now possesses steric hindrance with substrate or the charges on groups active with substrate have been neutralized.

Although a comparatively intensive and prolonged immunization schedule with all antigenic materials was used, no demonstration of alteration in antienzyme specificity was observed. Recent work on the serology of various mammalian amylase antigens incorporated in incomplete Freund's adjuvant employed as few injections as necessary to avoid the possibility of decreased antibody specificity (20). The inactivated α-amylase used showed no ability to produce a neutralizing antibody against β-amylase (barley). Therefore, alteration of this α-amylase through acid inactivation apparently does not alter its structure sufficiently to promote this modification in its antigenic specificity.

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Belladonna Alkaloid Analysis by Partition Column Chromatography

By RALPH S. SANTORO and ANDREW R. MATZ

A sharp separation of total belladonna alkaloids (atropine, hyoscyamine, and scopolamine) from chlorpheniramine[1] and phenylpropanolamine[2] was effected by partition column chromatography which permitted the colorimetric determination of the total belladonna alkaloids. The column utilized a stationary phase of pH 7.0 buffer with moving phases of 1:1 cyclohexane-carbon tetrachloride for removal of the chlorpheniramine, and chloroform for the elution of the alkaloids. Phenylpropanolamine was retained on the column. The method was applied to pharmaceutical formulations containing the belladonna alkaloids in combination with chlorpheniramine maleate and phenylpropanolamine hydrochloride.

THE SOLANACEOUS alkaloids, atropine, hyoscyamine, and scopolamine, the well known parasympatholytic agents, have been the subject of intensive investigation evidenced by the body of literature that has grown about these substances. Analysis and/or assay methods have also been extensively reported. The two colorimetric methods most common to the reported assay procedures and used for the actual determination of the separated alkaloids are the Vitali-Morin reaction, in which a violet color is produced from the condensation of aromatic nitro-groups with acetone in the presence of potassium hydroxide (1–5), and the formation of an alkaloid or tertiary amine complex with sulfonic acid indicators (6–13). Conditions which affect the precision and accuracy of results are also discussed in the above references. These methods have been successfully adapted to the *in vivo* and *in vitro* determination of total alkaloid content.

More recently, methods have appeared in which paper chromatography has been employed for the resolution of the solanaceous alkaloids into their separate components, followed by quantitative determination with the above, or other appropriate methods (14–18). It should be mentioned that determinations using means other than color development have been used, *e.g.*, polarographic (19) and infrared (20), where conditions possible for their use were present.

The particular method chosen for the determination is governed in part by such factors as the accuracy and precision required; amount of constituent present coupled with the sensitivity of the assay method; the form encountered, *i.e.*, crude extract, purified chemical, or pharmaceutical formulation; and the amount and nature of interfering substances. However, the problem of the actual determination many times does not resolve itself into a consideration of method advantage, but into a more basic consideration: achievement of a satisfactory separation from interfering substances so that a quantitative determination can be made.

This paper describes a method in which the total belladonna alkaloids, hyoscyamine sulfate, scopolamine hydrobromide, and atropine sulfate, were separated by partition column chromatography from a pharmaceutical preparation containing chlorpheniramine maleate and phenylpropanolamine hydrochloride. The total alkaloids were then determined colorimetrically with methyl orange as the complexing agent.

EXPERIMENTAL

Reagents.—Cyclohexane, practical grade, b.p. 71–81°.

Chloroform and carbon tetrachloride, reagent grade.

Celite (Hy-Flo Supercel, Johns-Manville Co.).

Buffer solution pH 5.6. Dissolve 27.22 Gm. of potassium dihydrogen phosphate, reagent grade, in 1 L. of distilled water and adjust to pH 5.6 with 0.1 *N* sodium hydroxide.

Methyl orange reagent. Saturate 1 L. of pH 5.6 buffer with approximately 2 Gm. of methyl orange, reagent grade, and filter through Whatman No. 1 filter paper. Extract the filtrate repeatedly with chloroform until the chloroform layer is colorless.

Buffer solution pH 7.0. Dissolve 5.42 Gm. of sodium dihydrogen phosphate, reagent monohydrate, and 16.36 Gm. of dibasic sodium phosphate, reagent heptahydrate, in 1 L. of distilled water and adjust to pH 7.0 with either 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide.

Standard solutions of alkaloids. Dry samples of hyoscyamine sulfate N.F., atropine sulfate U.S.P., and scopolamine hydrobromide U.S.P. overnight at 105°. Store the dried chemicals in a desiccator.

Stock standard. Weigh 0.869 Gm. of hyoscyamine sulfate, 0.175 Gm. of atropine sulfate, and

Received July 28, 1961, from Smith Kline & French Laboratories, Philadelphia 1, Pa.

Accepted for publication March 14, 1962.

The authors wish to acknowledge the assistance of Joseph J. Fitzpatrick and E. Lee Haines who contributed to the experimental part of this paper. We also wish to express our gratitude to Joseph Carpenter, Mary Maculate, and Martin De Porres for their guidance and help.

[1] 2-[*p*-Chloro-α-(2-dimethylamino-ethyl)benzyl]pyridine.

[2] α-(1-Aminoethyl)benzyl alcohol.

0.092 Gm. of scopolamine hydrobromide into a 100-ml. volumetric flask. Dissolve and dilute to volume with pH 5.6 buffer.

Intermediate working standard. Dilute 10 ml. of the stock standard to 500 ml. with pH 5.6 buffer.

Working standards. Dilute 12.5- and 25-ml. aliquots of the intermediate working standard to 100 ml. with pH 5.6 buffer. These standards represent 0.140 mg./5 ml. and 0.285 mg./5 ml. belladonna alkaloids.

Apparatus.—Klett-Summerson photoelectric colorimeter.

Chromatographic column. Approximately 19 mm. × 350 mm. with adapter equipped with a stopcock for controlling rate of flow (Ace Glass catalog No. 6261).

Rinco rotating evaporator.

Preparation of the Calibration Curve.—Transfer a 5.00-ml. aliquot of each of the two working standards to 125-ml. separators. To a third separator, transfer a 5.00-ml. aliquot of pH 5.6 buffer to serve as a reagent blank. Add 3.00 ml. of methyl orange reagent from a pipet to each funnel. Extract with 5–15-ml. portions of chloroform, collecting the chloroform extracts in a 100-ml. volumetric flask containing 15 ml. of 95% ethanol, through a pledget of cotton previously wet with chloroform. When 1-min. shake-outs are used, the chloroform layer should be essentially colorless by the fifth shake-out.

Read the color intensity of each of the standards on the Klett-Summerson photoelectric photometer, using a No. 42 filter and pH 5.6 buffer as the zero. Subtract the reagent blank from the readings obtained, and calculate the standard factor for each of the two standards by dividing the standard weight by the corrected reading. Take the average of the two. Each factor should not vary more than ±3% from the average factor.

The standard factor should be calculated daily or whenever new buffer is used.

Assay Procedure.—Accurately weigh a finely ground portion of the sample containing 0.5 to 0.7 mg. of total alkaloids into a 2-oz. mortar, add 1 ml. pH 7.0 buffer, and grind until a uniform slurry is obtained. Add 3 Gm. of Celite and 2 ml. of pH 7.0 buffer to the slurry. Regrind until the Celite is uniformly wet and homogeneous in texture.

In a second 2-oz. mortar, prepare an additional 3 Gm. of Celite by adding 3 ml. of pH 7.0 buffer and grinding until the physical appearance is as described above. Place a small mat of glass wool in the bottom of a glass chromatographic column. Transfer about 2.5 Gm. of the prepared Celite into the column and tamp firmly, but gently, with a suitable length of wood dowling.

Transfer all of the Celite containing the sample into the column: first, by scraping the mortar with a metal spatula after the loose bulk has been transferred, and adding the scrapings to the column; second, by adding the remaining Celite from the second mortar into the sample mortar, grinding the Celite around the bottom and sides of the mortar, and adding the grindings to the column as described in the initial step above. Retamp the column until the outside walls have a uniform porosity. Cap the column with a mat of glass wool.

Elute the column with 100 ml. of a mixture of 1:1 carbon tetrachloride and cyclohexane. After about 10 ml. of the mixture has been collected, stop the flow of solvent and allow the column to equilibrate for 15 minutes, then continue until elution has ceased. Discard the mixture. Add 80 ml. of chloroform to the column and collect the eluant in a 100-ml. flat-bottom boiling flask with a standard taper 24/40 ground-glass neck. Vacuum evaporate the collected chloroform to dryness, using a rotating evaporator at room temperature. Pipet 10 ml. of pH 5.6 buffer into the flask and heat gently on a steam bath until solution of the residue is effected.

Transfer a 4-ml. aliquot of this solution into a 125-ml. separator and proceed as directed above under preparation of the calibration curve, beginning with the addition of the methyl orange reagent. Run a second 4-ml. aliquot as the sample blank using 3.00 ml. of pH 5.6 buffer in place of the methyl orange reagent. Run a reagent blank using 4.00 ml. of pH 5.6 buffer in place of the sample. Calculate the total alkaloids by

$$\frac{[\text{Klett reading} - (\text{sample blank} + \text{reagent blank})] \times \text{standard factor} \times 100}{\text{sample weight (Gm.)} \times 4} =$$

mg. total belladonna alkaloids/Gm.

Modified Vitali Method.—The use of methyl orange for the colorimetric determination was adequate since the concentration of alkaloids in the samples was relatively large. The sensitivity of the methyl orange method can be improved by reconcentrating the extracted indicator into acid (21), but because the Vitali-Morin reaction has a greater sensitivity, its use would be indicated in samples containing a concentration of alkaloids below the sensitivity of normal indicator methods. Calibration curves on the mixed alkaloids were run by the modified Vitali method (2), and Beer's law adherence was obtained down to 0.023 mg. (Fig. 1).

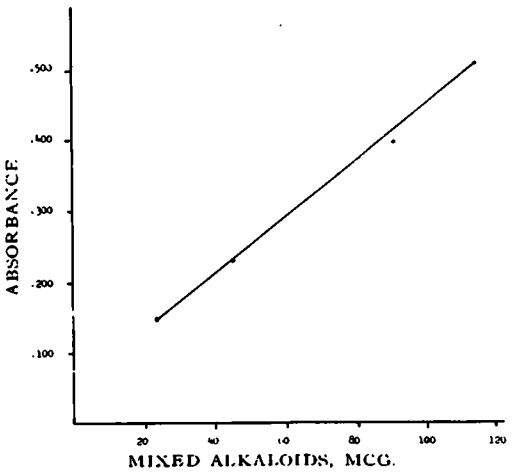


Fig. 1.—Modified Vitali method calibration curve of mixed alkaloids.

RESULTS AND DISCUSSION

Solvent partition experiments were run on hyoscyamine sulfate, chlorpheniramine maleate, and phenylpropanolamine hydrochloride at various pH conditions by equilibrating a known amount of chemical between equal volumes of the two phases. The amount remaining in the buffer phase was then determined by ultraviolet absorption. These experiments indicated that the conditions optimum to the separation of chlorpheniramine from hyoscyamine were extraction with cyclohexane at a pH of 7.0 (Table I). With the use of pH 7.6 buffer, 87% of the chlorpheniramine was extracted into the cyclohexane, but a loss of 3% hyoscyamine was encountered. Below pH 7.0, the extractability of both substances dropped markedly.

A suitable solvent extraction method was developed in which the chlorpheniramine was first removed by multiple extractions with cyclohexane from pH 7.0 buffer, followed by extraction of the alkaloids with chloroform. However, the results were uniformly high due to traces of chlorpheniramine remaining in the buffer phase, and increasing the number and volume of cyclohexane extractions served to reduce, but not eliminate, the positive error.

The distribution coefficient K for chlorpheniramine was calculated from

$$K = \frac{w_1/v}{(w - w_1)l} \qquad \text{(Eq. 1)}$$

where v is the volume in ml. of pH 7.0 buffer (phase I), w is the weight in Gm. of dissolved substance extracted with l ml. of cyclohexane (phase II), and w_1 is the solute remaining in phase I after the extraction (22). The chlorpheniramine remaining in the buffer after any given number of extractions is then

$$W_n = w\left(\frac{Kv}{Kv + l}\right)^n \qquad \text{(Eq. 2)}$$

Since the reaction of methyl orange is a molecular ratio of 1:1 in compounds having one basic amine function (23), the estimated error due to chlorpheniramine in the methyl orange reaction can be expressed as

$$E = \frac{w_n}{w_a} \times \frac{MW_1}{MW_2} \qquad \text{(Eq. 3)}$$

where w_a is the weight of alkaloids originally present with an average molecular weight of MW_1, and MW_2 is the molecular weight of chlorpheniramine maleate. From the data (Table I), K for chlorpheniramine maleate is 0.449, and calculating the estimated error after six extractions yields 10.5%. Beyond this number of extractions, simple solvent separation

Table I.—Partition Experiments with pH 7.0 Buffer

	Per Cent in Buffer Phase		
	Hyoscyamine Sulfate	Chlorpheniramine Maleate	Phenylpropanolamine Hydrochloride
Chloroform	58	...	100
Carbon tetrachloride	95	10	100
Petroleum ether	100	34	100
Cyclohexane	100	31	100

Table II.—Partition Column Recovery of Mixed Alkaloids

Determination No.	Substances Added, mg. Chlorpheniramine Maleate	Phenylpropanolamine Hydrochloride	Mixed Alkaloids	Recovery, mg.	Recovered, %
1	10.0	125.0	0.599	0.587	98.0
2	10.0	125.0	0.599	0.587	98.0
3	10.0	125.0	0.599	0.602	100.5
4	10.0	125.0	0.599	0.619	103.3

Table III.—Data from Pharmaceutical Formulations

Experiment	Theoretical Content	Initial Assay	Temperature Condition, °C.	Assay after Storage Time, mo. 1	2	3	4	6	9	12
A	0.400 mg./capsule	...	RT	...	...	...	0.395	...	...	0.403
B	0.250 mg./capsule	...	RT	0.256	0.240	0.237	0.231	0.240	0.242	0.238
		...	37°	0.259	0.244	...	...	...	...	...
		...	5°	...	0.244	...	...	...	...	...
C	0.250 mg./capsule	...	RT	0.264	0.249	...	...	...	...	...
		...	37°	0.253	...	...	...	...	...	...
		...	5°	0.251	...	...	...	...	...	...
D	0.630 mg./Gm.	0.610	...	...	...	...	...	...	...	...
E	0.250 mg./capsule	0.249	RT	0.245	...	...	...	...	...	...
F	0.250 mg./capsule	0.259	...	...	...	...	...	...	...	...
G	0.250 mg./capsule	0.251	...	...	...	...	...	...	...	...
H	0.610 mg./Gm.	0.570	...	...	...	...	...	...	...	...

was not only impractical, but showed a significant loss of hyoscyamine on known solutions due to the systemic errors inherent in the type of separation.

In order to extract the buffer exhaustively and effect the complete removal of chlorpheniramine, the technique of partition column chromatography was utilized for the separation. Recovery of the mixed alkaloids was quantitative in the presence of chlorpheniramine maleate and phenylpropanolamine hydrochloride present in the same ratio theoretically found in the samples (Table II). The mixed alkaloids in this study are susceptible to hydrolysis to form tropine, epoxy tropine, and tropic acid. Tropine and epoxy tropine react with methyl orange in the same fashion as the intact alkaloids; tropic acid does not react nor interfere with the determination.

The hydrolytic products were tested through the procedure with the result that the methyl orange blank and the hydrolysis product readings were the same. It was concluded that the degradation products were retained on the column and the method could be used as a stability procedure. The method was applied to samples of various pharmaceutical formulas for initial assay and stability determinations. The results from these experiments are listed in Table III.

Although cyclohexane was initially selected for the removal of chlorpheniramine, the mixture of cyclohexane and carbon tetrachloride was developed as the eluting solvent for two reasons: (*a*) cyclohexane caused a slower elution than desired, and (*b*) it did not remove many of the wax excipients normally associated with pharmaceutical formulations. The subsequent elution with chloroform extracted these excipients along with the alkaloids and caused hard waxy residues after the chloroform evaporation which tended to occlude the alkaloids in the pH 5.6 buffer solution.

The solvent mixture overcame the difficulties experienced with cyclohexane. The use of carbon tetrachloride itself as the eluting agent would be ideal from the standpoint of chlorpheniramine and wax removal, but the alkaloids also partition. Assays in which carbon tetrachloride was used gave low results, and decreasing the volume in an attempt to prevent the loss gave high and erratic results. The mixture selected, along with the two-stage feature of the column, accomplished a sharp separation with little interfering residue from the chloroform step.

It was found that the precision of the results was improved by equilibrating the column before each elution. The equilibration consisted of stopping the flow of eluant after 5–10 ml. had been collected and allowing the column to stand 15–20 minutes before elution was recommenced. This procedure was followed for both elutions. Time limitation necessitated the stopping of many assays during the elution with cyclohexane-carbon tetrachloride and the columns were allowed to stand overnight without affecting the results. Another advantage observed with equilibration and associated with the first elution was the further diminution of waxy residues in the chloroform eluate. Samples which were not finely ground before being incorporated in the column showed inconsistent results even with prolonged equilibration times, and indicated that equilibration was not a substitute for a properly prepared sample.

SUMMARY

A column partition separation of the total belladonna alkaloids, hyoscyamine sulfate, atropine sulfate, and scopolamine hydrobromide, from chlorpheniramine maleate and phenylpropanolamine hydrochloride is described in which the stationary phase is pH 7.0 buffer and the moving phases are 1:1 cyclohexane-carbon tetrachloride, for the removal of chlorpheniramine, and chloroform for the elution of the alkaloids. Phenylpropanolamine does not elute with either solvent system.

The total alkaloids were determined colorimetrically using methyl orange as the complexing agent. A modified Vitali procedure is recommended for determinations in which the alkaloid content is below the sensitivity of indicator methods.

Factors governing the selection of column partition chromatography as the method of separation, choice of the eluting solvents, advantage of equilibration, and necessity for proper sample preparation are also discussed.

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Interaction of Carrageenan and Other Hydrocolloids with Alkaloids II

Equilibrium Dialysis Studies

By HORACE D. GRAHAM and LINNIE B. THOMAS†

The extent of binding of the alkaloids, quinine hydrochloride, hyoscyamine hydrochloride, caffeine, and theophylline by the hydrocolloids carrageenan, furcellaran, sodium carboxymethylcellulose, gum tragacanth, sodium alginate, pectin, locust bean gum, and gum acacia was studied. Data were obtained employing varying pH levels of the reaction system and temperatures of 4, 27, and 37°. A Langmuir-type plot of the data to obtain the limiting binding capacity of the hydrocolloids at infinitely high concentrations of the alkaloids, indicates that quinine hydrochloride was bound to the greatest extent by most of the hydrocolloids used. The order of binding was as follows: carrageenan > sodium carboxymethylcellulose > furcellaran > sodium alginate. Locust bean gum, pectin, gum tragacanth, and gum acacia bound this alkaloid considerably less. Approximately the same trend was exhibited with hyoscyamine hydrochloride, whereas caffeine and theophylline were bound to a much smaller, but nonetheless significant, extent than the other alkaloids tested.

IN A PREVIOUS communication, Graham and Thomas (1) demonstrated that several natural and some synthetic hydrocolloids are precipitated by alkaloids of different classes. However, in some cases, no visible precipitation was noted although, on a structural basis, significant interactions could have occurred. Such subtle reactions can be studied best by equilibrium dialysis, a technique which has been used extensively to elucidate interactions between proteins and other substances (2, 3), between anionic polysaccharides and detergents (4), and for studying several other reactions (5–13).

This report summarizes the data obtained from studies on the interaction of several alkaloids with hydrocolloids which are commonly used in pharmaceutical and food formulations. Evidence has been obtained that, even in cases where no visible turbidity was evident, considerable binding of the alkaloids by the hydrocolloids occurred.

EXPERIMENTAL

Materials

Alkaloids.—Quinine hydrochloride (2%), hyoscyamine hydrochloride (1%), caffeine (1%), and theophylline (0.5%).

All samples were prepared in double distilled water. Where necessary, solution was accomplished by gentle heating in a water bath. Representative alkaloids were selected on the basis of the following characteristics: Quinine hydrochloride: this alkaloid reacts strongly with most hydrocolloids producing visible turbidity in most cases. Hyoscyamine hydrochloride: at low concentrations, this alkaloid produces little or no visible turbidity when mixed with the hydrocolloids used (1); however, at high concentrations (2–4%) ready precipitation occurs, especially with some of the sulfated hydrocolloids. Caffeine and theophylline: these alkaloids do not produce visible turbidity with any of the hydrocolloids used.

Hydrocolloids.—These are listed in Table I. All samples were prepared and dialyzed as previously described (1). All calculations are expressed on a dry weight basis. The moisture content of each hydrocolloid was determined by drying each sample to constant weight in a vacuum oven at 80°.

Equipment

Beckman DU spectrophotometer, Beckman pH meter, model G, 30-ml. Pyrex, glass-stoppered test tubes, 50-ml. acid buret, Burrell wrist-action shaker, refrigerated room at 4°, dialysis membrane—27/32 dialysis tubing, seamless cellulose, Fisher Scientific Co.

Procedure

Preparation of the Dialysis Bags.—Twelve-inch strips of the dialysis membrane were cut with as much uniformity as possible and 12-in. lengths of string were used to tie the bags. The strips of dialysis membrane and string were thoroughly washed in tap water, soaked in, and finally rinsed several times with distilled water. Dialysis bags were constructed by tying one end as tightly as possible, placing the hydrocolloid dispersion in the bag and then tying the open end securely.

Dialysis and Determination of the Amount of Alkaloid Bound by the Hydrocolloids.—The experimental regimen was essentially that employed by Klotz, *et al.* (12), and more recently by Patel and Kostenbauder (10). A 25-ml. quantity of the stock solution containing 10–1000 mg. of the particular hydrocolloid was placed in the dialysis bag, the open end was tied securely, and the bag placed in a wide-

Received June 2, 1961, from the George Washington Carver Foundation, Tuskegee Institute, Ala.
Accepted for publication February 13, 1962.
† National Science Foundation undergraduate research participant.
This investigation was supported by a grant from the National Institutes of Health, Bethesda, Md.
The authors wish to thank the following persons and agencies: Dr. Leonard Stoloff of Marine Colloids, Inc., New York, N. Y., for samples of carrageenan; Miss Florence Griffin for technical assistance; and Meer Corporation, New York, N. Y., for powdered samples of gum tragacanth, locust bean gum, and gum arabic.

TABLE I.- LIMITING BINDING CAPACITY (μM./GM.) OF HYDROCOLLOIDS FOR VARIOUS ALKALOIDS

Hydrocolloids	Alkaloids: Quinine HCl	Hyoscyamine HCl	Caffeine	Theophylline
Carrageenan (SeaKem type 5)	303.0	189.0	58.8	54.6
Sodium carboxymethylcellulose	232.0	111.1	53.0	38.4
Furcellaran	200.0	142.8	44.8	37.0
Sodium alginate	200.0	140.0	40.0	35.0
Pectin	169.1	136.8	38.0	33.0
Gum tragacanth	88.0	46.0	26.0	30.4
Locust bean gum	50.5	44.4	38.0	33.0
Gum acacia	47.0	26.0	16.0	18.6

mouth bottle containing 75 ml. of the particular alkaloid. The bottles were placed on a Burrell wrist-action shaker and reaction allowed to take place for 36 hours, an interval sufficient for the attainment of equilibrium. Experiments conducted using 300-ml. Erlenmeyer flasks, 50 ml. of the hydrocolloid dispersion, and 50 ml. of outside solution gave essentially similar results. However, the use of wide-mouth bottles as described offers the possibility of keeping the bathing fluid in better contact with the bag surface. Moreover, during the shaking, such bottles are less subject to breaking as was experienced with the flasks in some instances.

At the end of the reaction period, the outside solution was poured into a 100-ml. volumetric flask and the contents made up to volume with double distilled water. One milliliter of this solution was then used for determination of the free alkaloid in the solution on the outside of the dialysis bag. In several instances, the solution inside the dialysis bag was also analyzed for its content of both free and total alkaloid. In such cases, the solution was also accurately made up to volume. A control, which differed from the primary bag only in that the former contained distilled water instead of hydrocolloid, was included. This permitted corrections for any alkaloid bound by the dialysis membrane.

The amount of alkaloid bound by a given quantity of hydrocolloid was calculated by subtracting the sum of the free alkaloid in the system and the alkaloid bound by the bag from the total amount of alkaloid added.

Methods Used in Determining the Amount of Free Alkaloid

Routinely, the amount of free alkaloid was determined by the colorimetric dichromate-sulfuric acid method of Graham and Thomas (14). Essentially, the procedure is as follows: 1 ml. of the solution to be assayed is placed in a Pyrex, ground glass-stoppered test tube, 1 ml. of a 5% solution of potassium dichromate is added, and the mixture is incubated for 5 minutes in a constant temperature water bath at 30°. At the end of this incubation period, 8 ml. of concentrated sulfuric acid is added, the tube is swirled gently to mix the contents well, and then placed in an ice water bath for 20 minutes. Duplicate control tubes containing all ingredients except the alkaloid are included and are treated similarly. The color developed is measured at 650 mμ with a Coleman Universal spectrophotometer, model 14, or with a Beckman DU spectrophotometer, and the amount of alkaloid present determined by reference to a standard curve.

Since the alkaloids used absorb strongly in the ultraviolet region, the concentration of alkaloid in the solutions both inside and outside the dialysis bag, after dilution with distilled water, was determined, in some cases by ultraviolet spectrophotometry using the Beckman DU spectrophotometer. Quinine hydrochloride was determined at 331 mμ (9), caffeine at 272 mμ (15), and theophylline at 276 mμ (16).

Under the experimental conditions employed, excellent agreement was obtained whether the concentration of alkaloid was determined only in the solution outside the dialysis bag or both in the solution inside and outside of the dialysis bag. Similar excellence in agreement of values obtained by analyzing both the solution inside the dialysis bag and the solution outside the dialysis bag, has been noted by Patel and Kostenbauder (10) and by Ehrenpreis (3). Additionally, as seen from Table II, good agreement in results were obtained by both the colorimetric dichromate-sulfuric acid method and the ultraviolet absorbance method. In view of these facts, the dichromate-sulfuric acid method was used routinely.

Equilibrium Dialysis Calculations

Essentially, the method of calculation and the assumptions involved are similar to those outlined by Patel and Kostenbauder (10) in their studies on the binding of parahydroxybenzoic acid esters by Tween 80. In principle, the thin semipermeable

TABLE II.- AMOUNT OF ALKALOIDS BOUND BY HYDROCOLLOIDS AT 27° AS DETERMINED BY THE DICHROMATE-SULFURIC ACID (DS) METHOD AND BY THE ULTRAVIOLET (UV) SPECTROPHOTOMETRIC METHOD

Hydrocolloid	μm. of Alkaloid Bound per Gm. of Hydrocolloid at 27°: Quinine Hydrochloride DS Method	Quinine Hydrochloride UV Method	Caffeine DS Method	Caffeine UV Method	Theophylline DS Method	Theophylline UV Method
Carrageenan (SeaKem type 5)	256.0	262.0	40.0	42.2	48.6	48.0
Sodium carboxymethylcellulose	206.0	216.0	33.6	35.6	36.4	35.8
Furcellaran	198.0	189.4	38.0	40.4	29.6	29.0
Sodium alginate	175.0	186.2	26.8	29.6	24.8	24.2
Pectin	150.1	153.2	20.0	24.5	23.2	24.8
Gum tragacanth	88.0	74.6	26.0	24.8	21.6	20.7
Locust bean gum	50.0	48.2	25.0	26.2	23.4	22.9
Gum acacia	42.0	43.4	14.4	15.4	18.0	17.6

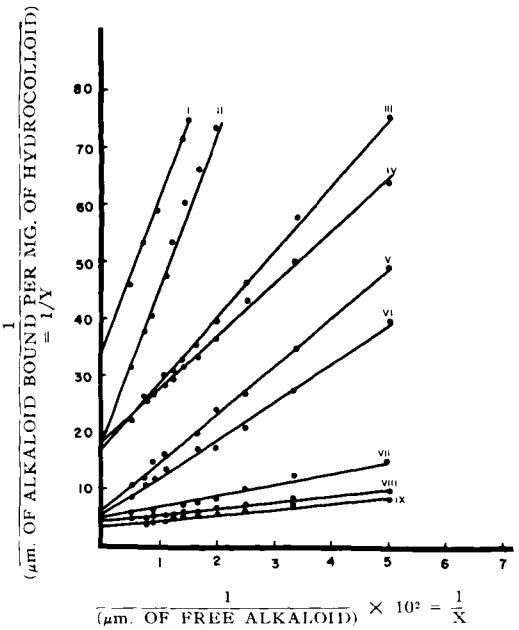


Fig. 1.—Plot of $1/y$ *vs.* $1/x$ for limiting binding capacity of hydrocolloids for various alkaloids, data from studies done at 27° in aqueous solutions. I, Locust bean gum-theophylline; II, sodium carboxymethylcellulose-caffeine; III, carrageenan-caffeine; IV, carrageenan-theophylline; V, furcellaran-hyoscyamine hydrochloride; VI, carrageenan-hyoscyamine hydrochloride; VII, sodium alginate-quinine hydrochloride; VIII, sodium carboxymethylcellulose-quinine hydrochloride; IX, carrageenan-quinine hydrochloride.

membrane permits free passage of the alkaloid but not of the hydrocolloid. The alkaloid placed on the outside of the bag will diffuse inwardly to react with the hydrocolloid placed on the inside of the bag. At equilibrium, the activity of the alkaloid will be equal on both sides of the membrane. For a sufficiently dilute solution it can be assumed that the concentration of the alkaloid on both sides of the membrane will be essentially the same. Therefore, if the macromolecule is placed on one side of the membrane and the concentration of alkaloid on the opposite side is determined, it becomes possible to determine the amount of free or unbound alkaloid in equilibrium with the hydrocolloid. The procedure employed is outlined symbolically: let A_t = total alkaloid added; let A_b = alkaloid actually bound by the hydrocolloid; let A_m = alkaloid bound by membrane; let $A_{f.o.}$ = free alkaloid outside of bag; let $A_{f.i.}$ = free alkaloid inside of bag = $A_{f.o.}$ Then, $2A_{f.o.}$ = total free alkaloid in system. Then $A_b = A_t - (2A_{f.o.} + A_m)$. In these calculations, the Donnan effects were neglected.

Influence of Temperature and pH on the Binding Process

In order to characterize the reactions more thoroughly, the influence of temperature and pH on the binding of alkaloids by the hydrocolloids was investigated.

A constant quantity of the particular hydrocolloid was allowed to equilibrate for 36 hours against an excess of each alkaloid according to the general procedure at 4, 27, and 37°. For the experiments at 4°, all reagents were preincubated at this temperature; the dialysis systems were set up and removal of aliquots for determination of the free alkaloid on the outside was done in the cold room. This precluded any experimental errors due to temperature changes which would result through removal of the flasks from the cold room.

Possible variations in the extent of binding at 27° caused by differences in the pH of the system were investigated by dialyzing 200 mg. of each hydrocolloid against an excess of each alkaloid. The alkaloids and hydrocolloids were suspended in 0.02 *N* phosphate buffers of varying pH levels. At the end of the dialysis period, the pH of the solutions inside the bag and outside the bag was checked with a Beckman model G pH meter. Quinine hydrochloride precipitates readily in the presence of salts, hence the quinine hydrochloride system was not investigated.

RESULTS AND DISCUSSION

Estimation of the Degree of Binding from the Equilibrium Dialysis Data.—Variations in the degree of binding of the various alkaloids by the hydrocolloids are shown in Table I and Fig. 1. The limiting or maximum binding capacity per unit of hydrocolloid at infinitely high levels of the alkaloids was ascertained by plotting the reciprocal of the amount of alkaloid bound per mg. of hydrocolloid (i/y) *vs.* the reciprocal of the amount of free alkaloid present (i/x). The y intercept is the reciprocal of the limiting quantity of the alkaloid bound per mg. of hydrocolloid. This approach has been used extensively by Kennon and Higuchi (9) and Patel and Kostenbauder (10) in studying the interaction of drugs with polyelectrolytes.

Figure 2 is a plot of the amount of alkaloid bound *vs.* the free alkaloid in the system at 27° in aqueous solutions. Carrageenan, carboxymethylcellulose, furcellaran, sodium alginate, and pectin bound quinine hydrochloride and hyoscyamine hydrochloride to a considerably greater extent than gum tragacanth, locust bean gum, and gum acacia. The so-called xanthine alkaloids, caffeine and theophylline, when mixed with the hydrocolloids used, produced no turbidity whatsoever. However, as shown by Fig. 2 and Table I, binding occurred. In defense of this observation, the work of Guttman and Athalye (17) may be cited. Here, quite stable complexes were formed between riboflavin and caffeine and theophylline. They postulated that the imidazole ring of the xanthine nucleus must be strongly involved. As in their studies, it may be postulated that although hydrogen bonding could possibly provide a primary linkage between the interactants, reinforcement by secondary van der Waals interactions involving other parts of the molecule could also have occurred. Studies on interactions of xanthine molecules with bovine serum albumin by Eichman, *et al.* (18), also indicate the capability of complex formation by caffeine and theophylline with organic molecules.

It is evident that the complexes formed by caffeine and theophylline with the hydrocolloids used are weaker than those complexes formed by quinine hydrochloride and hyoscyamine hydro-

chloride with the same hydrocolloids. This is particularly so with carrageenan, furcellaran, sodium carboxymethylcellulose, sodium alginate, and pectin, in which cases the interaction is primarily a salt formation.

Between 4° and 27°, the amount of each alkaloid bound by the hydrocolloids varies slightly. However, at 37° considerably greater binding was exhibited between the alkaloids quinine hydrochloride and hyoscyamine hydrochloride and the hydrocolloids carrageenan and sodium carboxymethylcellulose. See Table III. The increased binding at the higher temperatures could be due to a weakening of the sodium gegenion field around the polymer so that more drug-polymer interaction occurs. The reaction may be a complex one and besides ionic interactions, hydrogen bonding and secondary van der Waals interactions may occur. However, if van der Waals forces were the only or main factor involved, increased temperature would cause a decrease in interactions.

The interaction with caffeine and theophylline showed less variation with temperature within the range of 4–37°. This probably reflects the weak interactions which occurred as contrasted with quinine hydrochloride and hyoscyamine hydrochloride.

As seen from Table IV, the pH of the system had no marked effect on the extent of binding of the alkaloids by carrageenan. However, beyond either

TABLE III.—INFLUENCE OF TEMPERATURE ON THE BINDING OF ALKALOIDS BY HYDROCOLLOIDS

Hydrocolloid	μm. Alkaloid Bound per Gm. of Hydrocolloid 4°	27°	37°
	Quinine Hydrochloride		
Carrageenan (SeaKem type 5)	248.4	253.8	401.2
Carboxymethylcellulose	200.1	205.0	250.8
Gum tragacanth	91.6	90.4	94.6
Locust bean gum	48.4	49.2	49.8
	Hyoscyamine Hydrochloride		
Carrageenan (SeaKem type 5)	112.6	118.0	162.8
Carboxymethylcellulose	97.4	96.2	140.6
Gum tragacanth	40.8	45.2	51.6
Locust bean gum	38.2	39.35	40.8
	Caffeine		
Carrageenan (SeaKem type 5)	40.0	42.2	43.4
Carboxymethylcellulose	33.0	32.6	37.5
Gum tragacanth	26.4	27.2	30.2
Locust bean gum	25.2	25.8	28.8
	Theophylline		
Carrageenan (SeaKem type 5)	37.2	38.0	37.6
Carboxymethylcellulose	36.0	37.2	36.8
Gum tragacanth	31.6	31.4	32.6
Locust bean gum	24.0	23.4	22.8

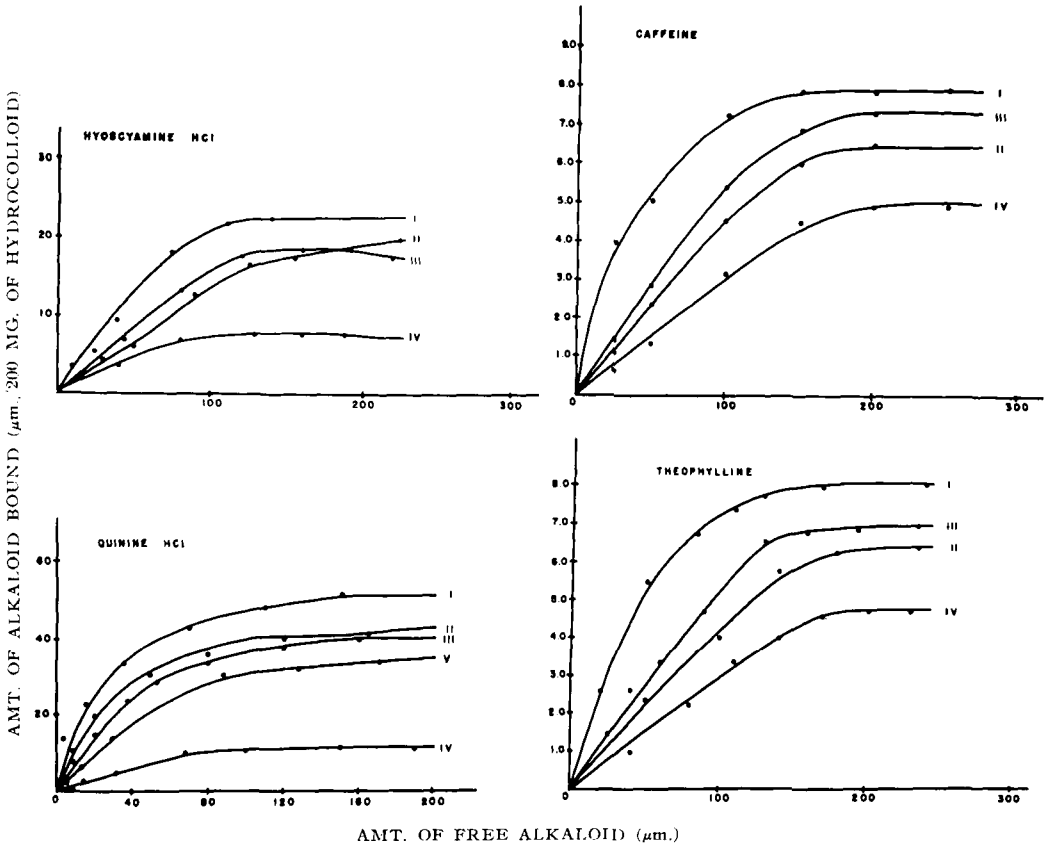


Fig. 2.—Binding of alkaloids by hydrocolloids at 27°. I, Carrageenan (SeaKem type 5); II, sodium carboxymethylcellulose; III, furcellaran; IV, locust bean gum; V, sodium alginate.

TABLE IV.—INFLUENCE OF pH ON THE BINDING OF ALKALOIDS BY HYDROCOLLOIDS AT 27°

	μm. Alkaloid Bound per Gm. of Hydrocolloid				
	pH 3.0	5.4	6.4	8.4	10.1
			Hyoscyamine Hydrochloride		
Carrageenan (SeaKem type 5)	105.8	118.6	120.0	115.6	110.0
Carboxymethylcellulose	84.6	94.2	97.6	93.8	90.0
Gum tragacanth	43.0	45.2	46.8	42.6	40.0
Locust bean gum	34.0	40.0	38.4	37.6	37.0
			Caffeine		
Carrageenan (SeaKem type 5)	40.6	42.8	40.3	41.4	39.8
Carboxymethylcellulose	30.4	33.0	32.8	30.6	32.5
Gum tragacanth	25.8	26.8	27.6	27.0	26.8
Locust bean gum	25.8	25.2	26.2	26.2	25.8
			Theophylline		
Carrageenan (SeaKem type 5)	37.8	40.0	38.4	39.2	39.0
Carboxymethylcellulose	37.4	35.0	35.4	34.6	38
Gum tragacanth	32.00	31.2	30.6	32.6	31.8
Locust bean gum	23.2	23.6	24.0	23.6	22.8

extreme, the stability of the hydrocolloid and, perhaps of the alkaloid, must be taken into serious consideration. Carboxymethylcellulose, in particular, would be relatively more severely affected than the sulfated hydrocolloids like carrageenan and furcellaran. At pH levels of about 3.0 or below, the ionization of sodium carboxymethylcellulose would be severely repressed and the hydrocolloid would precipitate (19). Data obtained at pH 3.0 for sodium carboxymethylcellulose may therefore be somewhat equivocal. As previously stated, studies in buffer solutions are complicated by precipitation of certain alkaloids, for example, quinine hydrochloride. Such side reactions, even if not visible, must not be ignored.

These studies bear significantly upon the problems which may arise when alkaloids and hydrocolloids are both incorporated into pharmaceutical and other preparations. Where rapid effects are required, the concentration of "free alkaloid" in the system and not the total alkaloid, is important. However, in many areas of nutritional (20) and pharmaceutical (21, 22) therapy, gradual or sustained release of the medicinal or nutritional constituent is desirable. Economy and convenience are two of the many advantages claimed for such sustained release. In this respect, hydrocolloids incorporated into the dosage forms may enhance the desired properties. Alternate effects, such as the inactivation of antimicrobial and other agents, could also be caused by the presence of such hydrocolloids as carrageenan and carboxymethylcellulose, as suggested by Eisman, *et al.* (23).

If binding data are available, it will be possible to approximate and perhaps determine the required concentration of a particular alkaloid or alkaloid-like agent necessary for eliciting a particular physiological function in the presence of specific hydrocolloids incorporated into the medicating mixture for emulsifying or other effects. Wagner (22), in an excellent review entitled "Biopharmaceutics: Absorption Aspects," paid critical attention to the interaction effects of adjuvants, such as gum acacia, when they are used in tablets and other preparations. Alterations in the surface area of the drug, if in solid forms, and inhibition of absorption through formation of a less soluble compound were cited as possibilities. The opposite of these effects may also be possible, hence the significance of such interactions in formulation work should not be overlooked.

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Relationship between *In Vitro* Dissolution Rates, Solubilities, and LT_{50}'s in Mice of Some Salts of Benzphetamine and Etryptamine

By W. MOROZOWICH, T. CHULSKI, W. E. HAMLIN, P. M. JONES, J. I. NORTHAM, A. PURMALIS, and J. G. WAGNER

Salt formation of benzphetamine and etryptamine as a potential means of obtaining sustained release and/or prolonged action was studied by measuring the median lethal time (LT_{50}) in mice and its relationship to *in vitro* dissolution rates and solubilities. Both the LT_{50} and LD_{50} increase as the *in vitro* dissolution rate, determined at pH 7.2, decreases. An empirical relationship was found between the LT_{50} and the *in vitro* dissolution rate at pH 7.2. The experimental data suggest that salt formation is a potentially useful means of approaching development of sustained- or prolonged-action dosage forms of certain drugs.

THE PURPOSES of this study were: (*a*) to investigate salt formation as a means of obtaining sustained release and/or prolonged action, and (*b*) to investigate relationships between toxicity data and *in vitro* dissolution rates and solubilities. The relative toxicities of a series of salts of a drug reflect the rate of absorption providing the salt-forming agents are relatively nontoxic. When absorption is rate-limited by dissolution of the salt in the gastrointestinal tract, as will be the case with slowly soluble salts, the toxicity of a slowly dissolving salt will most probably be lower than that of a more rapidly dissolving salt. A similar concept was applied by Nelson (1) in which the clinical effects noticed with various theophylline salts were explained on the basis of dissolution rates. Becker and Swift (2) showed that sustained-release properties of drug resinates could be evaluated by observing the median lethal time of death (LT_{50}) of the drug resinate. Greater sustained or slow release of drug provided an increased LT_{50} and an increased LD_{50} resulting from slower absorption. *In vitro* release of drug from other resinates (2, 3) and from sustained-release capsules[1] (4) was found to correlate with toxicity and blood levels in animals. Shenoy, Grice, and Campbell (5) found the determination of the LD_{50} of a sustained-release formulation to be a very useful method of assessing sustained-release preparations; however, the limitation of the method was the inability to distinguish between sustained and/or slow release and nonavailability of drug. They found that supposed sustained-release products which failed to show sustained release by means of urinary excretion studies in human subjects also showed no sustained release in LT_{50} studies in animals.

The compounds selected for this study were etryptamine[2] and benzphetamine,[3] both of which are basic amino compounds with apparent pKa values in water of 8.8 and 9.3, respectively. Both amine bases have low water solubilities but form highly soluble hydrochlorides.

EXPERIMENTAL

Preparation of Salts.—Salts of the amine drugs were prepared by (*a*) the reaction of the amine hydrochloride with an alkali salt of an acid in aqueous or aqueous-organic solvent mixtures, or by (*b*) reaction of the amine base with a free acid in an appropriate solvent. Elemental analysis and equivalent weight confirmed the stoichiometry of the salt.

Determination of LT_{50} and LD_{50}.—Each salt was ground to a fine powder in a mortar, suspended in 1.0% carboxymethylcellulose by means of a tissue grinder, and intubated orally into ten starved mice. The amount of salt administered was equivalent to that amount which just produced 100% death in an acute toxicity study. In the benzphetamine series it was that quantity equivalent to 400 mg./Kg. benzphetamine hydrochloride, whereas in the etryptamine series it was that quantity equivalent to 124 mg./Kg. etryptamine acetate. The death time of each animal was recorded to the nearest minute.

The LT_{50} was estimated as the time midway between the times to death of the fifth and sixth animals. This method is an alternative to that in which the intersection of the 50 percentile point with a plot of cumulative percentage mortality on log-probability graph paper is taken as the LT_{50} (2–5). This method was chosen since many sets of such mortality data are not truly log-normally distributed.

Determination of *in vitro* Dissolution Rates and Solubilities.—The solubilities were determined by: (*a*) equilibration of excess compound with water for

Received January 8, 1962, from The Upjohn Co., Kalamazoo, Mich.
Accepted for publication January 23, 1962.

[1] Spansule, trade-marked name of Smith Kline and French's sustained-release capsule.

[2] Etryptamine (Monase, as the acetate) is 3-(2-aminobutyl)indole.

[3] Benzphetamine (Didrex, as the hydrochloride) is (+)N-benzyl-N, α-dimethylphenethylamine.

24 hours at room temperature and determination of the amount in solution, or by (*b*) visual observation of the amount which just dissolves upon equilibration as above.

The dissolution rates were determined by the hanging pellet method of Nelson (6). The pellets were 13 mm. in diameter and were compressed at 20,000 p.s.i. The dissolution medium was 500 ml. of 0.1 *N* hydrochloric acid or 0.2 *M* tris(hydroxymethyl) aminomethane solution adjusted to pH 7.2 with hydrochloric acid and maintained at 37°.

RESULTS AND DISCUSSION

Tables I and II show the various salts of benzphetamine and etryptamine, respectively, arranged in order of increasing LT_{50}. The cumulative death data used in determining the LT_{50} are shown in Tables III and IV. Inspection of Tables I and II shows that the *in vitro* dissolution rates determined at pH 7.2 correlate much better with LT_{50} than do either the water solubilities or dissolution rates determined at pH 1. There is poor correlation of water solubility with dissolution rate, probably because the water solubility is an equilibrium condition at an uncontrolled pH whereas the dissolution rate is a dynamic property dependent upon many factors (7). The polygalacturonate salt of benzphetamine is an example of a salt with a very high water solubility but low dissolution rates. An accurate determination of the solubility of this salt was hindered by the high viscosity of the resultant solution. The seeming anomalously low dissolution rates of this salt are probably due to the high viscosity of the diffusion layer.

Some salts, notably the 8-nitrotheophyllinates, diliturates, bis-methylene-salicylates, and pamoates showed unusual pellet dissolution at pH 1.0. It was found that a layer of the insoluble acid, resulting from hydrolysis of the salt, was formed on the surface of the pellet while the protonated amine diffused through this barrier.

Only a few of the LD_{50}'s were determined (Table I). However, for those available there appears to be a qualitative relationship between LD_{50}, LT_{50}, and dissolution rates at pH 7.2; both the LT_{50} and LD_{50} increase as the dissolution rate decreases. It appears then that one should always use the most rapidly dissolving salt or other form of a drug in determining the intrinsic acute LD_{50} of the drug; conversely, the apparent acute toxicity may be decreased by employing a slowly dissolving form of the drug.

When the LT_{50} of each salt was plotted against the *in vitro* dissolution rate, determined at pH 7.2,

TABLE I.—BENZPHETAMINE SALTS

Salt	M.p., °C.	Equivalent Water Solubility,[a] mg./ml.	Equivalent Rate of Dissolution,[b] mg./cm.²/hr. pH 1.0	pH 7.2	LT_{50}[c]	90% Confidence Limits of LT_{50}[d]	Equivalent LD_{50},[e] mg./Kg.
Hydrochloride	131–133	434–781	564	542	10	8–12	174
					8	6–10	...
Diliturate	133–134	5.01	...	5.5	12	11–13	...
Polygalacturonate	...	...	3.7	9.5	14.5	13–18	193
8-Nitrotheophyllinate	173–174	0.66	3.26	0.86	16.5	14–22	225
Barbiturate	203–205	4.36	94.5	4.30	27	21–322	248
Methylene-bis-salicylate[f]	89–91	0.15	0.013	0.20	30.5	13–139	...
2,4-Dihydroxybenzoate	73–75	12.9	51	1.6	98	30–1117	...
Pamoate[f]	101–103	0.06	3.6	0.07	804	169–1375+	278

[a] Equivalent water solubility = solubility of the salt × $\frac{\text{mol. wt. of the amine base}}{\text{mol. wt. of the salt}}$. [b] Equivalent dissolution rate = apparent dissolution rate × $\frac{\text{mol. wt. of the amine base}}{\text{mol. wt. of the salt}}$. [c] Median lethal time estimated as the time midway between the times to death of the fifth and sixth (of 10) mice. [d] Estimated from the time to death of the third and eighth (of ten) mice to die. [e] Equivalent LD_{50} = LD_{50} of the salt × $\frac{\text{mol. wt. of the amine base}}{\text{mol. wt. of the salt}}$. [f] The stoichiometry of these salts is 2 amine:1 acid.

TABLE II.—ETRYPTAMINE SALTS

Salt	M.p., °C.	Equivalent Water Solubility,[a] mg./ml.	Equivalent Rate of Dissolution,[b] mg./cm.²/hr. pH 1.0	pH 7.2	LT_{50}[c]	90% Confidence Limits of LT_{50}[d]
Acetate	165–166	88.8	336	264	3	2–3
Free base	104–105	0.51	97.8	24.5	4	4–5
8-Chlorotheophyllinate	212–214	3–5	157	40.9	4.5	4–5
Barbiturate	257–258	5–10	112	11.4	12.5	10–14
Pamoate[b]	136–144	0.03–0.12	7.9	0.098	111	37–124
8-Nitrotheophyllinate	214–217	0.25	5.56	0.49	209	119–277

[a, b, c, and d] See footnotes in Table I.

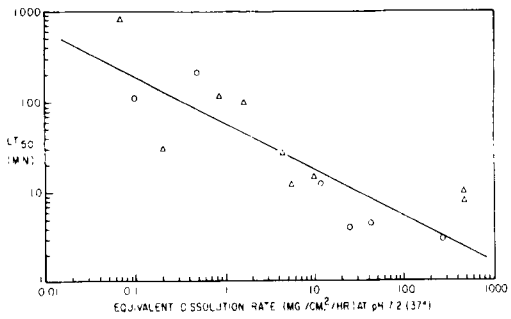


Fig. 1.—A plot of LT_{50} in mice against equivalent dissolution rate at pH 7.2 for etryptamine and benzphetamine salts. Δ, Benzphetamine salts; O, etryptamine salts.

on log-log graph paper, the points appeared to be linearly distributed. Estimation of the least squares regression line indicated the slope of the line was approximately −0.5. Figure 1 is such a plot with the solid line having a slope of −0.5. It follows that for the salts studied, the LT_{50} is inversely related to the square root of the equivalent *in vitro* dissolution rate determined at pH 7.2. This treatment is similar to that of Sturtevant, *et al.* (4), in which linear relationships were found between the logarithm of ΔLT_{50} and the logarithm of the *in vitro* release rates of various sustained-release amphetamine bead preparations.

SUMMARY AND CONCLUSIONS

1. Series of salts of benzphetamine and etryptamine were prepared.

2. The water solubility, *in vitro* dissolution rates determined in aqueous media having a pH of 1.0 and 7.2, and the median lethal time of death (LT_{50}) were determined for each salt. The LD_{50} (mice) of five of the benzphetamine salts, following oral administration, were also determined.

3. The experimental data show that the times to death of mice following a lethal dose and the LD_{50}'s of the various salts are related to the rates of dissolution of the salts as determined by a standardized *in vitro* test.

4. Regression analysis indicated that the LT_{50} is inversely related to the square root of the equivalent *in vitro* rate of dissolution determined at pH 7.2 for the salts studied.

5. The water solubilities and the *in vitro* rates of dissolution determined at pH 1.0 did not correlate nearly as well with the toxicity data as did the *in vitro* rates of dissolution determined at pH 7.2.

TABLE III.—CUMULATIVE DEATH DATA OF BENZPHETAMINE SALTS

	Times, to Death of Mice for Benzphetamine, min.							
% Mortality	Hydrochloride	Dilitrate	Polygalacturonate	8-Nitrotheophyllinate	Barbiturate	Methylene bis-Salicylate	2,4-Dihydroxybenzoate	Pamoate
100	11	35	60	209	...	195	7200	...
90	...	21	20	...	...	194	...	...
80	10	13	18	22	322	139	1117	...
70	...	12	17	21	28	41	162	1375
60	8	...	15	18	27	39	161	...
50	...	...	14	15	...	22	35	234
40	7	11	13	14	25	21	33	206
30	6	...	...	...	21	13	30	169
20	5	...	11	...	...	...	13	162
10	3	10	9	...	19	...	10	121

TABLE IV.—CUMULATIVE DEATH DATA FOR ETRYPTAMINE SALTS

	Times to Death of Mice for Etryptamine, min.					
% Mortality	Acetate	Base	8-Chlorotheophyllinate	Barbiturate	Pamoate	8-Nitrotheophyllinate
100	4	6	5	15	276	308
90	3	5	...	14	153	280
80	...	4	...	...	124	277
70	...	...	...	13	114	249
60	...	...	...	...	112	233
50	...	...	4	12	110	186
40	2	...	...	10	47	175
30	...	...	...	...	37	119
20	...	...	...	...	34	...
10	...	3	...	9	...	115

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Excretion Patterns of Phenothiazine-S^{35} Compounds in Rats

Effect of Change in Structure on Metabolism

By THOMAS L. FLANAGAN, JACK H. NEWMAN, ALFRED R. MAASS, and EDWARD J. VAN LOON

The excretion patterns of five S^{35}-labeled phenothiazines were compared in rats following the oral administration of pharmacologically active doses. Substitution in the 2 position in the phenothiazine ring did not have any apparent effect upon the S^{35} excretion pattern. Differences in the structure of the side chain did have an effect upon the mode of S^{35} excretion. The urinary S^{35} excretion decreased and the fecal S^{35} excretion correspondingly increased as the side-chain structure was changed from 3-dimethylaminopropyl to 3-dimethylamino-2-methylpropyl to 4-methyl-1-piperazinylpropyl. At a dosage level of 9.0 mg. of free base/Kg. in rats, the urinary S^{35} excretion following the oral administration of compound II [2-chloro-10-(3-dimethylaminopropyl)phenothiazine-S^{35} hydrochloride] was twice as great as the urinary S^{35} excretion following the oral administration of compound III [10-[3-(4-methyl-1-piperazinyl)-propyl]-2-trifluoromethylphenothiazine-S^{35} dihydrochloride]. The former compound contained a chlorine atom in the 2 position of the phenothiazine nucleus, while the latter contained a trifluoromethyl grouping at this position. The increased dosage level (9.0 mg. of free base/Kg.) did not appreciably change the S^{35} excretion pattern of compound IV [10-(3-dimethylamino-2-methylpropyl)phenothiazine-S^{35} tartrate] and compound V [10-(3-dimethylamino-2-methylpropyl)-2-trifluoromethylphenothiazine-S^{35} hydrochloride].

ALTHOUGH there are reports in the literature on the urinary excretion pattern of chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)-phenothiazine hydrochloride] there are few comparative studies of the excretion patterns of various phenothiazines. Christensen and Wase (1), using mice as the experimental animals, were the first to report on the urinary and fecal excretion of chlorpromazine-S^{35}. In a preliminary communication Fyodorov and Shnol (2) reported that, following the oral administration of chlorpromazine-S^{35} (aminazine-S^{35}) to rats, 16–17% of the radioactivity was excreted in the urine while approximately 76% was excreted in the feces. In other communications Fyodorov (3, 4) reported on the fate of three phenothiazine compounds in the organism: aminazine-S^{35}, promazine-S^{35}, and chlormeprazine-S^{35}. In his studies with aminazine-S^{35}, Fyodorov found a high concentration of radioactivity in the bile following oral administration. He reported (5) that a large part of the aminazine did not enter into the general blood stream but circulates in a closed circuit: intestine, portal vein, liver, bile, and back to the intestine being eliminated gradually in the feces.

In our laboratories it was shown that, following the intraduodenal administration of chlorpromazine to anesthetized dogs, the bile contained a high percentage of the drug and its metabolites (6). In similar experiments in dogs with three S^{35}-labeled phenothiazines, it was found that the phenothiazines were excreted in large but varying amounts *via* the biliary route. In light of these results it was decided to study the excretion patterns of five phenothiazine-S^{35} compounds in normal rats to see what effect changes in nucleus substitution and/or in the side chain would have upon urinary and fecal excretion following oral administration. Because of the large biliary excretion of the phenothiazines, it was recognized that the fecal pattern of each drug would not be a measure of unabsorbed drug but rather an indication of the amount of phenothiazine entering into the closed circuit system.

The structures of the phenothiazine-S^{35} compounds utilized are shown in Fig. 1.

Received January 17, 1962, from the Research and Development Division, Smith Kline & French Laboratories, Philadelphia 1, Pa.
Accepted for publication March 27, 1962.
The authors express their appreciation to Dr. Richard L. Young who collaborated in the initial stages of the present study and to John Pauls for the statistical interpretation of the experimental data.

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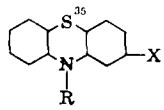
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	R =	X =	Salt
Compound I:	$-CH_2-CH_2-CH_2-N(CH_3)_2$	—Cl	·HCl
Compound II:	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	—Cl	·2HOOCCH=CHCOOH
Compound III:	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	—CF_3	·2HCl
Compound IV:	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	—H	·½ $C_4H_6O_6$
Compound V:	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	—CF_3	·HCl

I = 2-Chloro-10-(3-dimethylaminopropyl)phenothiazine-S^{35} hydrochloride
II = 2-Chloro-10-[3-(4-methyl-1-piperazinyl)-propyl]phenothiazine-S^{35} dimaleate
III = 10-[3-(4-Methyl-1-piperazinyl)-propyl]-2-trifluoromethylphenothiazine-S^{35} dihydrochloride
IV = 10-(3-Dimethylamino-2-methylpropyl)phenothiazine-S^{35} tartrate
V = 10-(3-Dimethylamino-2-methylpropyl)-2-trifluoromethylphenothiazine-S^{35} hydrochloride

Fig. 1.—Structures and nomenclature of phenothiazine-S^{35} compounds used in the studies.

EXPERIMENTAL

Preparation of S^{35} Phenothiazines

The phenothiazine-S^{35} compounds were prepared in this laboratory by Dr. D. W. Blackburn (7).

Dosage

Study 1.—Five groups of male, albino rats, 10 rats per group, averaging between 250 to 300 Gm., were used. Each group received a single oral dose of a solution of a labeled drug by stomach tube. In the initial study the drugs were administered at dosage levels (Table I) based upon the pharmacologically active dose, *i.e.*, the dose that blocks avoidance response. The rats were fasted overnight prior to drug administration. Immediately following drug administration, each animal was placed in an individual metabolism cage. Food and water were allowed *ad libitum*. Urine and fecal collections were made at 24-hour intervals for 96 hours. Each day the animals were transferred to clean cages.

Study 2.—The purpose of the second study was to compare the excretion pattern of phenothiazine-S^{35} compounds in nonfasted rats with that of fasted rats. Three of the labeled drugs were administered by stomach tube to groups of nonfasted rats at the same dosage levels as study 1. After administration, the study was completed as described under study 1. However, only the radioactivity of the urine specimens was determined.

Study 3.—To compare the effect of drug administration at the same dosage level upon excretion, the five labeled phenothiazines were administered by stomach tube to groups of 10 rats at a dosage level of 9.0 mg. of free base content of drug per Kg. of body weight. The animals were not fasted and the experiment was completed as described in study 2.

Oxidation and Counting Procedures

The urine specimens were made to volume and a suitable aliquot was counted in a Packard Tricarb liquid scintillation spectrometer, model 314 X, using a phosphor system which contained 80 Gm. of naphthalene and 5 Gm. of 2,5-diphenyl-oxazole dissolved in a mixture of toluene, 1,4-dioxane, and absolute alcohol (1:1:0.6) in a 1 L. volume.

Each 24-hour fecal collection was placed in a 100-ml. Kjeldahl flask containing 4–5 glass beads and 50 ml. of 8 *N* nitric acid. The fecal samples were soaked overnight and then digested to remove all carbonaceous material. The samples were evaporated to 5 ml. or less and cooled to room temperature. Nine milliliters of a digestion mixture consisting of 1:1 8 *N* nitric acid and perchloric acid (60% Baker's analyzed reagent) was added to each sample and digestion continued until bumping occurred. If the digest was not clear, an additional 3 ml. of the digestion mixture was added and the digestion continued until a clear solution was obtained.

After cooling, the contents of the flasks were transferred to 150-ml. beakers and the pH of the solutions adjusted to 3.0 with alkali. The $S^{35}O_4$

TABLE I.—URINARY AND FECAL EXCRETION OF RADIOACTIVITY FOLLOWING A PHARMACOLOGICALLY ACTIVE DOSE OF DRUG

Compound	Dosage, (mg./Kg.)	Fasted or Nonfasted	Urinary Extraction of Radioactivity: % of Dose Excreted 0–24 hr. Range	0–24 hr. Av.	24–48 hr. Range	24–48 hr. Av.	48–72 hr. Range	48–72 hr. Av.	72–96 hr. Range	72–96 hr. Av.	Total Range	Total Av.
I	10	F	24.2–48.3	31.6	3.2–12.6	5.7	0.4–1.4	0.9	0.1–0.3	0.2	32.2–52.2	38.4
II	5	F	6.7–13.9	10.4	0.7–3.0	1.6	0.4–1.4	0.7	0–0.7	0.4	8.6–16.7	13.1
III	1.25	F	5.5–14.2	8.3	0.4–1.0	0.7	0.2–0.3	0.2	0.02–0.10	0.08	6.2–15.3	9.3
IV	5	F	14.7–31.5	24.3	0.6–6.4	2.3	0.3–1.2	0.5	0.02–0.20	0.10	16.9–37.8	27.2
V	2.5	F	13.0–21.3	17.9	1.1–1.7	1.4	0.4–0.8	0.6	0.2	0.2	14.7–23.4	20.1
I	10	N	28.1–39.3	34.1	3.5–5.5	4.6	1.8–3.2	2.4	1.3–2.0	1.6	37.1–49.3	42.8
III	1.25	N	7.3–11.6	8.8	1.1–2.2	1.5	0.3–0.54	0.46	0.14–0.36	0.27	9.8–14.1	11.1
V	2.5	N	13.1–23.4	18.6	1.2–2.6	2.1	0.4–0.7	0.6	0.19–0.29	0.24	14.9–26.3	21.5
			Fecal Excretion of Radioactivity									
I	10	F	20.6–43.2	28.4	13.2–32.2	21.6	2.3–6.0	3.9	0.4–1.1	0.6	50.9–59.1	54.5
II	5	F	11.0–86.4	66.8	3.1–70.2	17.8	1.3–20.1	4.8	0.6–2.0	1.1	85.1–95.9	90.5
III	1.25	F	52.6–78.6	71.8	4.5–31.9	12.9	1.7–3.5	2.3	0.7–1.2	0.9	79.5–93.4	87.9
IV	5	F	42.5–76.5	60.1	3.0–18.7	9.2	0.6–5.2	1.6	0.3–1.5	0.5	60.5–80.6	71.4
V	2.5	F	54.7–75.7	61.9	11.2–19.0	14.9	1.9–5.6	3.6	0.9–1.6	1.2	77.4–89.7	81.6

TABLE II.—URINARY EXCRETION OF RADIOACTIVITY FOLLOWING THE ADMINISTRATION OF THE SAME DOSE OF FREE BASE TO NONFASTED RATS

Compound	Dosage: As Salt	Dosage: As Free Base	% of Dose Excreted 0–24 hr. Range	0–24 hr. Av.	24–48 hr. Range	24–48 hr. Av.	48–72 hr. Range	48–72 hr. Av.	72–96 hr. Range	72–96 hr. Av.	Total Range	Total Av.
I	10	9	28.1–39.3	34.1	3.5–5.5	4.6	1.8–3.2	2.4	1.3–2.0	1.6	37.1–49.3	42.0
II	14.6	9	14.7–28.0	20.6	1.5–5.2	3.2	0.7–3.3	1.8	0.2–2.7	1.3	19.1–39.1	27.0
III	10.6	9	9.4–12.5	10.8	1.0–2.1	1.4	0.26–0.48	0.36	0.13–0.24	0.17	11.4–15.4	12.8
IV	11.2	9	25.8–37.2	30.3	1.7–3.9	2.4	0.20–0.48	0.33	0.09–0.30	0.14	27.8–41.9	33.1
V	9.9	9	19.9–22.7	21.2	2.0–5.0	3.4	0.41–0.82	0.55	0.11–0.35	0.19	23.7–27.2	25.4

was precipitated as $BaS^{35}O_4$ with 5% barium chloride solution in the usual manner. Subsequently the precipitates were filtered, dried, weighed, and counted on a gas flow counter having a micromil window (Nuclear-Chicago 192). The samples were corrected to infinite thinness. In all the studies only total radioactivity was measured. As stated previously, fasted and nonfasted rats were used in separate experiments.

DISCUSSION AND RESULTS

The urinary and fecal S^{35} excretion values obtained on the experimental animals following the administration of a pharmacologically active dose of each drug are given in Table I. The urinary S^{35} excretion in fasted and nonfasted animals was similar for each compound following the adminstration of compounds I, III, and V (Table I). However, slightly higher recoveries were obtained in the nonfasted animals over a 96-hour collection period. In the first two studies in which the animals were administered the pharmacologically active dose of drug, the average urinary recoveries of the S^{35} compounds fell into three ranges: (*a*) The highest S^{35} average recovery (38–43%) was obtained from compound I which had a dimethylaminopropyl side chain. (*b*) The lowest average urinary S^{35} recoveries, 13 and 10%, were obtained with compounds II and III, respectively, which have a 4-methyl-1-piperazinyl propyl side chain. (*c*) The urinary S^{35} excretion of the two compounds which have a dimethylamino-2-methylpropyl side chain, compounds IV and V, was intermediate, 27 and 20%, respectively.

In the comparison of compounds II with III and IV with V, the data show that the S^{35} compound which was administered at the higher dosage level (Table I) gave the higher S^{35} concentration in urine. Because of the difference in dosage levels, no attempts were made to correlate the differences in the urinary S^{35} excretion based upon substitution in the 2 position of the phenothiazine ring.

The fecal S^{35} excretion patterns also fell into three ranges as shown in Table I, and they were exactly opposite to the urinary S^{35} patterns. The fecal excretion of compounds IV and V was 82 and 71%, respectively. This 11% difference was significant at the $P = 0.01$ level ($t = 4.15$). However, no correlation was made of this difference because there was a difference in dosage level, and the respective compounds were excreted to the extent of 62 and 60% in the first 24 hours.

When the fecal S^{35} excretion data were compared from the two sets of compounds which have an identical ring structure and differ only in substitution in the side chain, compounds I with II and compounds V with III, it was seen that the two compounds which contain a 4-methyl-1-piperazinyl propyl side chain (II and III) were primarily excreted in the feces.

In the third study the five phenothiazines were administered at the same dosage level of free base (9 mg./Kg.) to nonfasted rats. When the dose of compound II was increased from 5 mg. of compound/Kg. to 14.6 mg./Kg. (equivalent to 3.1 and 9.0 mg. of free base/Kg., respectively), the urinary S^{35} excretion was increased from 13 to 27% based

upon the administered dose of drug. A threefold increase in dose caused a twofold increase in urinary S^{35} output (Table II). An eightfold increase in the dose of compound III caused only a slight increase in the urinary S^{35} excretion of this compound.

The difference in structure between compounds II and III other than the salt form is in the substitution in the 2 position in the phenothiazine nucleus; compound II contains a chlorine atom, while compound III contains the trifluoromethyl grouping. This was the only case in which substitution in the 2 position of the nucleus appeared to have an effect upon the excretion pattern.

Although the dosages of compounds IV and V were increased two- and fourfold, respectively, the urinary S^{35} excretion of these compounds was increased only slightly (Table II). The increase in urinary S^{35} excretion following the increase in dose of compound II was similar to that obtained with promazine-S^{35}, 10-(3-dimethylaminopropyl) phenothiazine hydrochloride. Fyodorov (3), following the oral administration of promazine-S^{35} to rats at dosage levels of 12–20 mg./Kg., reported that the urine contained 40% of the administered S^{35} activity. At dosage levels of 50 mg./Kg. of promazine-S^{35} to rats intragastrically by stomach tube, Walkenstein and Seifter (8) reported that approximately 65% of the S^{35} dose was excreted in the urine. Compound I (chlorpromazine-S^{35}) was administered at only one dosage level due to a limited supply of this compound.

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Relation of Cathartic Activity to Structural Modifications of Ricinoleic Acid of Castor Oil

By M. S. MASRI, L. A. GOLDBLATT, F. DeEDS, and G. O. KOHLER

Castor oil and certain of its derivatives were tested in rats to elucidate the specific structural configuration of castor oil responsible for its cathartic action. The results indicate that the hydroxyl function on carbon-12 and the double bond between carbons 9 and 10 of the ricinoleic acid moiety are essential for the cathartic action, as evidenced by the loss of activity upon either masking of the hydroxyl group or hydrogenation of the double bond. On the other hand, the naturally occurring *cis* configuration of the double bond is not essential for this action, as evidenced by the retention of the activity upon elaidinization to the *trans* configuration.

CASTOR OIL, which is obtained from the seeds of *Ricinus communis*, consists mainly of the triglycerides of an unsaturated hydroxy fatty acid, ricinoleic acid, having the formula $C_{17}H_{32}$-(OH)COOH. Castor oil acts as a cathartic for man although considerable amounts may be absorbed and utilized when fed as part of the diet of many animals. Paul and McCay (1) reported 92.1% utilization by rabbits and 99% utilization by sheep when castor oil was fed to the extent of 6%. Stewart and Sinclair (2) fed adult rats a diet containing 48.4% castor oil and found that the oil was readily metabolized with no evidence of catharsis. Perkins, *et al.* (3), recently reported that weanling rats fed 10% triricinolein "gained as much weight as those fed corn oil (10%) diets" and found (private communication) no indication of catharsis.

Received March 12, 1962, from Western Regional Research Laboratory of Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.
Accepted for publication April 16, 1962.
The authors are indebted to Dr. William E. Ribelin for the histopathological examination and to Dr. R. H. Wilson for useful discussions.

Since ricinoleic acid differs from oleic acid, generally considered to be the most plentiful as well as the most widely distributed of all fatty acids in natural fats, only in having a hydroxyl group at carbon-12, it was of interest to investigate the effect of chemical modifications of the functional groups of ricinoleic acid upon the cathartic activity. The modifications planned included esterification of the hydroxyl group, hydrogenation of the double bond, and elaidinization, that is, conversion of the naturally occurring *cis* to the *trans* isomer. Materials tested included castor oil and the hydrogenated, elaidinized and acetylated oil. Castor oil, although it is predominantly the triglyceride of ricinoleic acid, is not a single chemical compound. Therefore, the investigation was extended to include highly purified methyl ricinoleate and its elaidinized, hydrogenated, and acetylated derivatives. The structural formulas for these four compounds are given in Fig. 1.

EXPERIMENTAL

Material and methods.—Castor oil grade AA,

upon the administered dose of drug. A threefold increase in dose caused a twofold increase in urinary S^{35} output (Table II). An eightfold increase in the dose of compound III caused only a slight increase in the urinary S^{35} excretion of this compound.

The difference in structure between compounds II and III other than the salt form is in the substitution in the 2 position in the phenothiazine nucleus; compound II contains a chlorine atom, while compound III contains the trifluoromethyl grouping. This was the only case in which substitution in the 2 position of the nucleus appeared to have an effect upon the excretion pattern.

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EXPERIMENTAL

Material and methods.—Castor oil grade AA,

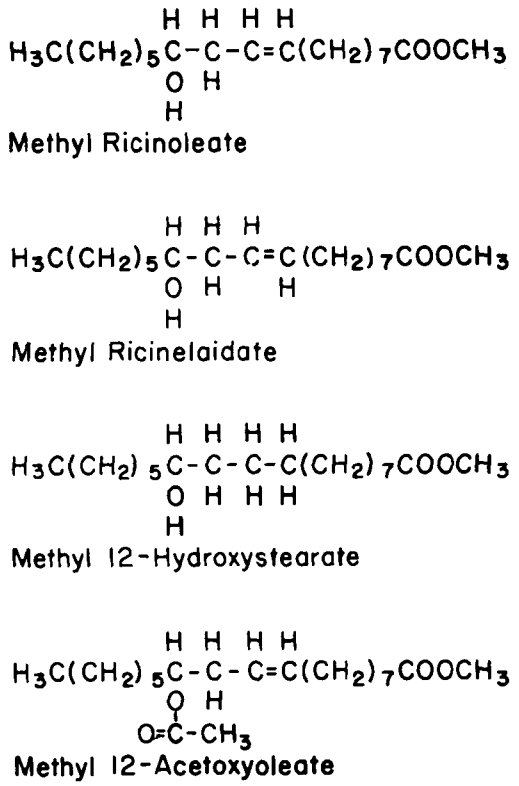


Fig. 1.—Formulas of methyl esters tested.

hydroxyl value 165, a commerical product,[1] was employed.

Hydrogenated Castor Oil.—Fifty grams of castor oil in 250 ml. ethanol was hydrogenated in the presence of 0.3 Gm. of platinum dioxide at a pressure of 55–39 p.s.i. gauge in a Parr hydrogenator. After 5 hours the theoretical amount of hydrogen had been taken up and absorption of hydrogen ceased. Hydrogenation was begun at room temperature of 24° and the temperature rose spontaneously to 29°. However, as the hydrogenation progressed the saturated product began to crystallize from the solution and it was necessary to raise the temperature by external heating to above 50° to keep the product in solution. After hydrogenation was discontinued the catalyst was filtered off and the solvent removed with a rotary evaporator at reduced pressure at a temperature below 70°. The hydrogenated oil, obtained in practically quantitative yield as an almost colorless white solid, melted at 83.5° to 85.2°.

Elaidinized Castor Oil.—Castor oil was elaidinized with a relatively small quantity of nitrite-nitric acid solution as the isomerization agent essentially according to the procedure of McCutchon, *et al.* (4), for the elaidinization of methyl ricinoleate. *To 150 Gm. castor oil maintained at 60°* was added with rapid stirring (magnetic stirrer) 10 ml. of 2 *M* sodium nitrite and 6.7 ml. of 6 *M* nitric acid. The heating and stirring was continued for 4 hours, after which the mixture was poured into 500 ml. of hot distilled water contained in a separator. The water layer was drawn off and the elaidinized oil washed again with 500 ml. hot distilled water. The oil, which was orange-brown in color and solidified below 50°, was then extracted with 500 ml. of a mixture of ethyl ether and Skellysolve F (1:1). The solution was dried with sodium sulfate and decolorized by percolating through a 200-Gm. column of alumina previously wetted with ether-Skellysolve F. The colorless eluate was evaporated to dryness at reduced pressure. The waxy, nearly colorless product melted at 48.9° to 51.0° and showed characteristic absorption in the infrared region at 10.3 μ indicating about 80% conversion of the *cis* olefinic unsaturation to the *trans* (elaidinized) configuration.

Methyl Ricinoleate.—This ester was obtained by alcoholysis of castor oil, followed by fractional distillation of the crude esters according to the procedure of Swern and Jordan (5) and purified further by low temperature crystallization. A heart cut of the distillate which was already better than 98% pure, α_D^{26} + 4.68°,[2] was further purified by fractional crystallization from Skellysolve F-acetone. One hundred and fifty grams of methyl ricinoleate was dissolved in a mixture of 250 ml. Skellysolve F and 50 ml. acetone and cooled to −30°. An impure first crop, α_D^{26} + 4.57°, amounting to 12 Gm. was thus obtained. The filtrate was then cooled to −36° whereby a second crop amounting to 83 Gm. was obtained, α_D^{26} + 4.70°. A third crop obtained at −50° was again somewhat impure, α_D^{26} + 4.65°. Recrystallization of a portion of the second crop did not increase its optical activity and this material, liquid at room temperature, was used in the biological tests.

Ricinoleic Acid.—Ricinoleic acid was prepared by saponification of purified methyl ricinoleate, α_D^{26} + 4.68°, essentially according to the procedure of McCutchon, *et al.* (4).

Anal. Calcd. for $C_{18}H_{34}O_3$: Acid value and hydroxyl value 187.9. Found: acid value 183.2; hydroxyl value 181.3.

Methyl Ricinelaidate.—Methyl ricinoleate was elaidinized with a small quantity of nitrite-nitric acid solution as the isomerization agent and purified by crystallization from Skellysolve F-acetone followed by decolorization with carbon and recrystallization from acetone-water (4). The product thus obtained melted at 29.2° to 30.8°. The magnitude of the characteristic absorption in the infrared region at 10.3 μ indicated quantitative conversion of the *cis* to the *trans* configuration in the purified product.

Methyl 12-Hydroxystearate.—Methyl ricinoleate was hydrogenated in the same manner previously described for the hydrogenation of castor oil except that no external heating was required since the product remained in solution at all times. Using 50 Gm. methyl ricinoleate, 250 ml. 95% ethanol, and 0.2 Gm. platinum oxide at a pressure of 50–30 p.s.i. the calculated amount of hydrogen was absorbed in about 2 hours at 30° but shaking under hydrogen was continued for another hour. The catalyst was then filtered off and the product allowed to crystallize at −2° overnight. The precipitate was collected and recrystallized from

[1] We are indebted to the Baker Castor Oil Co. for a generous supply of castor oil.

[2] Rotations reported are those observed in a 10-cm. tube.

methanol, 5 ml. per Gm. at 2°. The product thus obtained was a white crystalline solid, m.p. 56.4° to 57.6°.

Methyl 12-Acetoxyoleate.—Methyl ricinoleate was acetylated by treating 62.4 Gm. (0.2 mole) of the ester with 320 ml. of an acetic anhydride-pyridine mixture (1:3) in a 1-L. round-bottom flask which was fitted with a reflux condenser and thermometer. The mixture was stirred and heated to 100° and maintained at this temperature for 2 hours. After cooling to 75°, 300 ml. of water was added, and the mixture was stirred rapidly at this temperature for 15 minutes. It was then cooled to room temperature, transferred to a separator, and extracted with 400 ml. of ether. The ether extract was washed four times with 500-ml. portions of 3 *M* hydrochloric acid, after which it was dried over anhydrous sodium sulfate. The recovered product was fractionally distilled at reduced pressure. The main fraction, 40 Gm. distilling at 154° at 0.23 mm., had $\alpha_D^{26} + 23.01°$. This liquid distillate was used for the biological tests.

Acetylated Castor Oil.—Castor oil (hydroxyl value 165) was acetylated in the same manner as methyl ricinoleate. The crude reaction product was extracted with ether and washed and dried but was not distilled. The 3.3 hydroxyl value of the product indicated that 98% of the hydroxyl groups originally present in the oil had been acetylated.

Biological Tests.—Young adult male albino rats from a colony maintained in our laboratory since 1931 were used. The rats were fed a diet previously described (6). For the determination of cathartic activity, the rats were fasted overnight but allowed free access to water. In the morning they were lightly anesthetized with ether and stomach-tubed with the test substances. Samples which are solid at room temperature were warmed and given in the melted state with or without warm corn oil as a diluent to overcome their tendency to crystallize at body temperature. The relatively high melting point of hydrogenated castor oil, in particular, necessitated the use of a relatively large volume of corn oil. Two pellets weighing about 15 Gm. of a commercial ration for rats and a water bottle were supplied immediately after stomach-tubing. The rats were housed individually in hanging-wire cages with soft tissue paper spread underneath. The bottom of each cage was 9.5″ × 8″ made from 2-mesh, 16-gauge galvanized wire cloth which allowed the feces to drop freely on the paper. The number of wet feces produced in 24 hours was taken as an index of catharsis (7). Feces were scored wet when they were so fluid as to be unformed and produced distinct brown stains on the tissue paper surrounding them. Also, wet feces, when allowed to dry, could not be brushed off without tearing the paper. When catharsis was evident almost all the wet feces were usually produced within a few hours of stomach-tubing and occasionally it was necessary to replace the tissue papers with fresh ones in order to facilitate counting of the wet feces. In one experiment in which castor oil constituted 10% of the diet, weanling instead of adult rats were used. They were observed for growth and catharsis over a period of 5 weeks. For this experiment the diet with the following percentage composition was employed: corn meal 53, sucrose 10, casein 17, salt mixture U.S.P. XIV 4, corn oil 4, adequate vitamin mixture triturated in dextrose 2, and castor oil 10.

Table I.—Cathartic Action of Test Substances in Rats

Material Stomach-Tubed	No. of Rats	Body wt., Gm. Mean ± S.E.	Wet Feces/Rat Mean	Wet Feces/Rat S.D.
Castor oil 0.5 ml.	32	152 ± 3	6.8	4.6
Castor oil[a] 0.5 ml.	20	173 ± 3	7.2	3.6
Elaidinized castor oil[a] 0.5 ml.	10	164 ± 2	7.7	3.2
Hydrogenated castor oil[b] 0.5 ml.	10	186 ± 8	2.1	1.8
Acetylated castor oil 0.6 ml.	20	165 ± 4	0.8	1.3
Ricinoleic acid 0.5 ml.	10	153 ± 2	3.6	4.7
Ricinoleic acid[a] 0.5 ml.	10	156 ± 5	4.7	4.9
Methyl ricinoleate 0.5 ml.	10	178 ± 8	6.5	3.5
Methyl ricinoleate[a] 0.5 ml.	10	174 ± 7	6.6	5.0
Methyl ricinelaidate 0.5 ml.	9	152 ± 4	5.5	3.3
Methyl ricinelaidate[a] 0.5 ml.	10	198 ± 7	6.1	3.6
Methyl 12-hydroxystearate[a] 0.5 ml.	10	168 ± 9	0.2	0.4
Methyl 12-acetoxyoleate 0.5 ml.	10	150 ± 6	0.9	1.1
Corn oil (control) 0.5 ml.	20	166 ± 3	0.1	0.4
Corn oil (control) 2.0 ml.	10	202 ± 4	1.3	2.2
Corn oil (control) 3.5 ml.	8	216 ± 9	3.3	2.8

[a] With 1.5 ml. corn oil. [b] With 3.0 ml. corn oil.

RESULTS AND DISCUSSION

The results obtained with stomach-tubed samples are shown in Table I. It is evident that castor oil, elaidinized castor oil, methyl ricinoleate, methyl ricinelaidate, and ricinoleic acid all produced catharsis under our conditions. On the other hand, hydrogenated castor oil, acetylated castor oil, methyl 12-hydroxystearate, and methyl 12-acetoxyoleate were without cathartic action. The apparent slight cathartic activity of the sample of acetylated castor oil tested may be due to its slight content of incompletely acetylated oil. It should be emphasized that the number of wet feces given in Table I represent only a rough but measurable index of the degree of catharsis. Although the individual wet feces differed with respect to size and degree of wetness, each was assigned the same numerical value of one. The subjective appraisal is therefore also important, and a strict statistical comparison of the numbers of wet feces is not intended. However, it should be stated that the subjective appraisal of the individual tests paralleled the conclusions to be drawn from the numerical values.

In the feeding experiment in which 10 four-week-old male rats were fed *ad libitum* the diet containing 10% castor oil for approximately 5 weeks, no catharsis was observed and growth was comparable to that obtained with a similar control group in which the castor oil was replaced by corn oil. The mean weight and its standard error of the rats receiving castor oil was 42.2 ± 0.6 Gm. at the start and 155.6 ± 4.6 Gm. at the end of the experiment.

The corresponding means for the control group were 42.2 ± 0.5 Gm. and 160.5 ± 4.1 Gm., respectively.

Gross examination at autopsy showed no abnormalites. Histopathological examination of the perirenal fat also showed no abnormalities.

The results indicate that either saturation of the double bond or masking of the hydroxyl group of the hydroxy-double bond system of ricinoleic acid nullifies the cathartic action. On the other hand, neither isomerization of the double bond from the *cis* to the *trans* configuration, nor conversion of the neutral triglyceride to the methyl ester or to the free acid, appears to affect the cathartic action to an appreciable extent. The fact that the highly purified (distilled and crystallized) methyl ricinoleate is very effective militates against the possibility that the cathartic action of castor oil may be due to a trace contaminant.

The quite different results obtained when castor oil was given to fasted rats by stomach tube or as a part of the diet is striking. In the latter case no catharsis was observed, although the daily food intake toward the end of the feeding experiment provided well over 1 Gm. of castor oil per rat. This is in agreement with the observations of others, *e.g.*, Stewart and Sinclair (2) found that no catharsis occurred even when castor oil was included in the diets of rats at levels as high as 48.4%. There is no ready explanation for this difference. The reported use of castor oil as an article of the diet in China (8), presumably without untoward effects, may be related to this phenomenon. It is noteworthy that when used as a cathartic, castor oil is usually taken on an empty stomach (9).

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Technical Articles

Water Vapor Sorption and Diffusion through Hard Gelatin Capsules

By W. A. STRICKLAND, Jr., and MARTHA MOSS

Gelatin contains appreciable amounts of water under normal environmental conditions. This water may be transferred to a hygroscopic powder contained in a gelatin capsule. This report describes a study of this water transfer and of the diffusion of water vapor through gelatin capsules.

THE TWO-PIECE hard gelatin capsule is a convenient single dose medicine container that prevents ill taste on swallowing and promptly dissolves on reaching the stomach. The development of high speed filling equipment and the availability of well made, low cost capsules has prompted renewed interest in capsules as a dosage form. Capsules are made from refined animal gelatin and may contain very small amounts of other substances such as dyes or preservatives; however, the physical properties of hard gelatin capsules are essentially the properties of the gelatin from which they were made. Gelatin normally contains 9 to 12% water, but the water content can vary from about 4 to about 16%, depending on the environment. It is conceivable that the water content of normal capsules may exercise a deleterious effect on the capsule content provided it can be transferred. In addition, atmospheric water vapor may diffuse through capsules if the content is hygroscopic. Gelatin cannot be completely dehydrated by normal desiccating procedures and, if low water content is achieved, the capsules become brittle and fracture easily; conversely, greater than normal water content causes

Received February 12, 1962, from the University of Arkansas Medical Center, School of Pharmacy, Little Rock.
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The results indicate that either saturation of the double bond or masking of the hydroxyl group of the hydroxy-double bond system of ricinoleic acid nullifies the cathartic action. On the other hand, neither isomerization of the double bond from the *cis* to the *trans* configuration, nor conversion of the neutral triglyceride to the methyl ester or to the free acid, appears to affect the cathartic action to an appreciable extent. The fact that the highly purified (distilled and crystallized) methyl ricinoleate is very effective militates against the possibility that the cathartic action of castor oil may be due to a trace contaminant.

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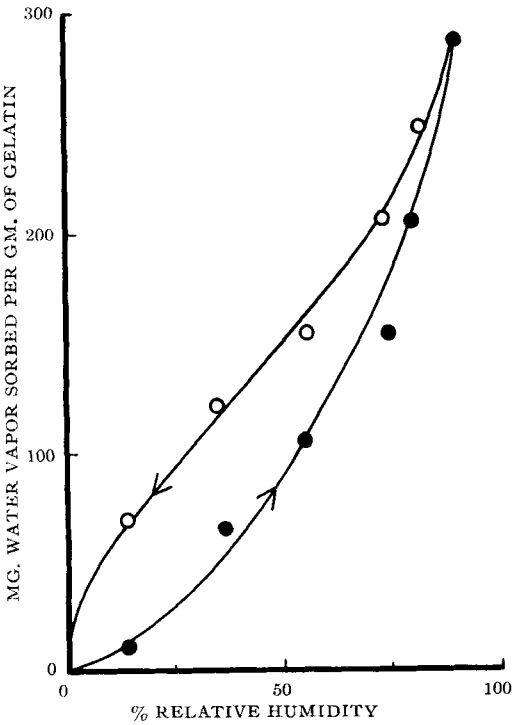


Fig. 1.—Typical sorption-desorption isotherm of crushed gelatin capsules in relative humidity chamber showing pronounced hysteresis of the desorption portion (open points) of the curve.

sticking and ultimate softening of capsules (1, 2).

This communication describes a study of water vapor transfer from capsules to hygroscopic powders and vice versa, and an apparatus used to study rate of water diffusion through gelatin capsules with typical results to show its application. A single lot of size 00 capsules (Parke, Davis and Co., Lot No. G5210-20) was used throughout this study.

EXPERIMENTAL

Sorption and Desorption of Water Vapor by Gelatin.—Isotherms showing the sorption and desorption of water vapor by gelatin were determined in relative humidity chambers by changing the relative humidity by increments at weekly intervals, as previously described (3), at 24°. Figure 1 shows a typical isotherm using crushed gelatin capsules (8 mesh) prepared by drying to constant weight over concentrated sulfuric acid. While the shape of the curve is subject to some variance with different gelatins, invariably there is pronounced hysteresis of the desorption portion of the curve. Perhaps the hysteresis is due to failure to achieve equilibrium, but it is reproducible and, therefore, significant in this work because desorption characteristics govern the availability of water for absorption by hygroscopic materials enclosed in capsules.

Water Vapor Transfer from Gelatin to Hygroscopic Medicinal Powders.—Transfer of water vapor from gelatin to hygroscopic powders, and vice versa, was studied by placing approximately 5 Gm. of accurately weighed samples of crushed gelatin capsules (8 mesh) and of hygroscopic powders (60 to 100 mesh) in aluminum foil dishes, about 5 cm. diameter, and bringing them to constant weight in relative humidity chambers maintained at the desired relative humidity by saturated salt solutions (4). Pairs of samples, one of gelatin and one of a hygroscopic powder, were placed in well closed containers of 1 L. capacity for 1 week at 21 ± 2°, after which time the gain or loss of sample weight was found by weighing. Table I gives typical data found in one such study.

Rate of Water Diffusion through the Capsule Wall.—The apparatus shown in Fig. 2 was devised to study rate of water diffusion through the capsule wall. Nitrogen gas, bubbled through a column of water to saturate it with water vapor, was passed through an equilibrating tube, then into the sample chamber at a constant rate of approximately 50 ml. per minute. The quartz helix springs, previously described (3), employed to determine the gain or loss of weight of sample capsules gravimetrically, had a maximum capacity of 2 Gm. at an extension of

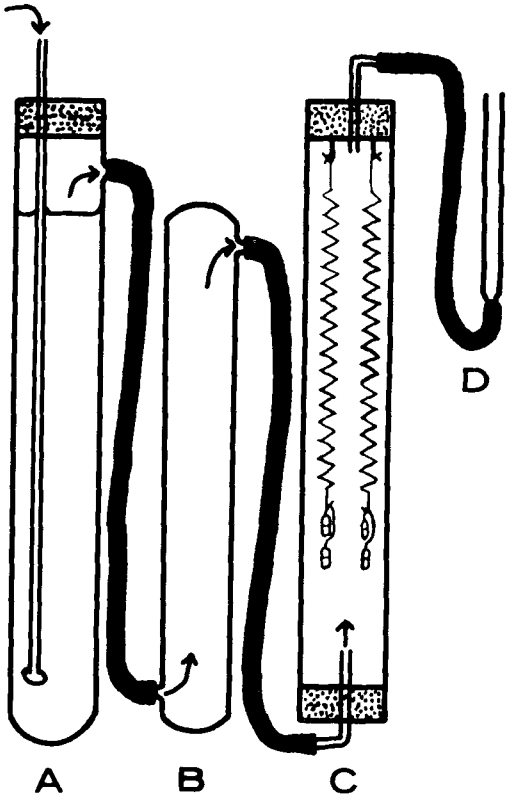


Fig. 2.—Diagram of apparatus used to determine lag time of water vapor diffusion through capsule walls. A, Water reservoir through which nitrogen was bubbled to saturate the gas; B, empty tube to assure temperature equilibrium and precise vapor saturation; C, sample chamber with sample capsules suspended from quartz springs; D, flow meter. The entire apparatus was immersed in a water bath maintained at 24 ± 0.2°.

TABLE I.—VAPOR PHASE TRANSFER OF WATER BETWEEN CRUSHED GELATIN CAPSULES AND PENTOBARBITAL SODIUM POWDER, U.S.P.

Material	Weight of Samples at Equilibrium (% Relative Humidity)	Wt. after 1 Week in Closed Containers (21°)	Net Gain or Loss, Gm.[a]
Pentobarbital sodium	4.9789 (0)	5.0380	+0.0925
Gelatin (8 mesh)	4.5047 (72)	4.4210	−0.0837
Pentobarbital sodium	5.1541 (72)	5.0426	−0.1115
Gelatin (8 mesh)	4.2911 (0)	4.4789	+0.1878

[a] The difference in gain and loss is accounted for by the atmospheric water vapor present and the end point relative humidity in the liter container.

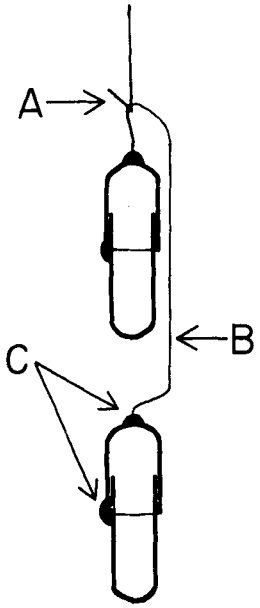


Fig. 3.—Pairs of capsules suspended from the lower hook of quartz spring, A; a small wire, B, was attached to capsules by spots of melted gelatin, C.

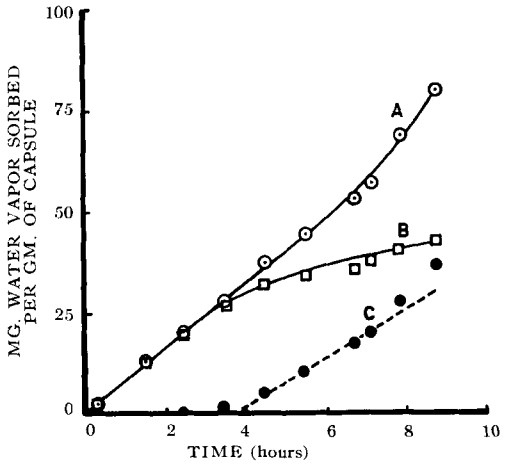


Fig. 4.—The result of a typical determination of water vapor diffusion through gelatin capsules. The weight gain, per Gm., of unfilled capsules, B, subtracted from the weight gain of starch filled capsules, A, yields the lag time of diffusion, C.

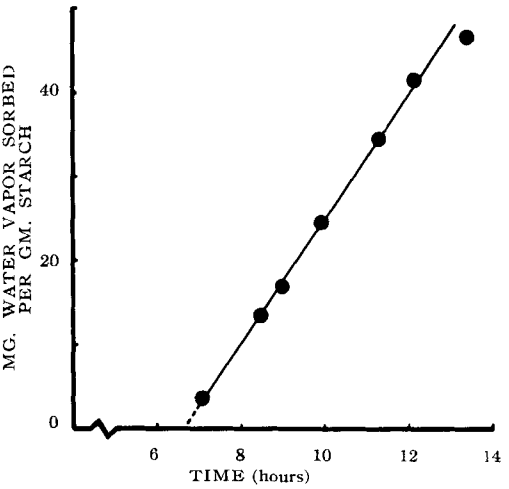


Fig. 5.—The lag in diffusion of water vapor through gelatin capsules filled with dried corn starch and coated with stearic acid.

approximately 1 cm. for each 51 mg. added weight. A cathetometer reading to 0.01 cm. was used to determine spring extension.

Sample capsules were suspended in pairs (Fig. 3) from the lower hook of the quartz spring. Capsules employed in this study weighed about 0.120 Gm. each and had a capacity of about 0.50 Gm. corn starch. After the larger section of the capsule was filled with a dried hygroscopic powder, the two parts of the capsule were gently pushed together and a spot of melted gelatin placed on the juncture to prevent the two sections from coming apart during experimental procedures. Glass beads were placed in the blank capsules to give a total weight near that of powder-filled capsules. Pairs of capsules were attached to a small wire, as shown in Fig. 3, then the assembled capsules were placed in a desiccator for 1 week or more and dried to constant weight prior to use. Figure 4 gives the results obtained when empty capsules and corn starch-filled capsules were placed in the apparatus and weight gain *vs.* time was determined. The time lag for water penetration through the capsule wall was about 4 ± 1 hours. Hygroscopic powders other than starch were used to fill capsules and yielded essentially the same results. Figure 5 depicts the results of determining the diffusion rate of water through capsules coated with stearic acid prepared by immersing capsules in a 2% carbon tetrachloride solution of stearic acid. The capsules appeared to be incompletely coated on visual inspection, which may account for the less than expected increase in lag time.

On occasion, primary softening or melting of capsule content at the juncture of the capsules was noted; however, in most determinations it appeared as if the juncture formed a good seal and the water

vapor diffused rather uniformly through the entire surface of the capsule.

CONCLUSIONS

The water content of gelatin capsules may be subject to desorption in the presence of hygroscopic materials. Relative hygroscopicity of the gelatin capsules and the filling materials determines the direction of vapor phase transfer of water in the closed capsule system. Gelatin capsules offer little protection to a hygroscopic content from atmospheric water vapor. Certain coatings may enhance this protective function, and the extent of protection may be evaluated in terms of prolonged diffusion lag time.

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Notes

Determination of Calcium Carbonate Moisture Content by a Radioisotope Technique

By MEI CHEN SCHNEITER†, WAYNE V. KESSLER, and JOHN E. CHRISTIAN

The transmission of beta particles through calcium carbonate samples with different moisture contents was studied. It was found that the transmission was linearly related to the moisture content.

IN MANY pharmaceutical manufacturing processes the moisture content of various materials is important. Conventional methods for moisture analysis are time consuming and delay further processing and subsequent production steps. Thus, a definite need exists for a rapid, simple method for moisture analysis. The objective of this investigation was to study the feasibility of determining the moisture content of pharmaceutical products using the beta-particle transmission technique. Although this technique has been used for determining the moisture content of certain materials such as wood (1) and paper (2), no reference to its use for moisture analysis of pharmaceutical products has been reported.

Calcium carbonate was selected for this study because it is a component of many pharmaceutical products. The transmission of the beta particles from a SrY-90 source through calcium carbonate samples with known moisture contents was measured. It was found that the transmission was linearly related to the moisture content.

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Accepted for publication March 16, 1962.
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This work was performed in part under contract with the Division of Isotopes Development, United States Atomic Energy Commission, Contract No. AT(11-1)-737.

EXPERIMENTAL

Radiation Counting Equipment and Radioactive Source.—A conventional decade scaler, a Geiger-Müller tube with a 1.4 mg./cm.^{2} end window, and a lead shield with a Lucite tube mount were used for the counter. The radioactive source was a $^3/_{16}$-in. diameter deposit of SrY-90 in the center of a $1^1/_4$-in. aluminum planchet having five concentric circular grooves on the bottom. The activity of the deposit was 218,000 c.p.m. at a distance of 2.4 cm. from the detector window. The deposit was covered with a thin coat of label glaze. The planchet was attached to an aluminum planchet holder which, in turn, was placed in the Lucite tube mount of the lead shield.

Preparation and Measurement of Samples.—Stock samples of wetted calcium carbonate were prepared from calcium carbonate powder (A.R. grade) and distilled water. For each stock sample, 100 Gm. of calcium carbonate was mixed with the

vapor diffused rather uniformly through the entire surface of the capsule.

CONCLUSIONS

The water content of gelatin capsules may be subject to desorption in the presence of hygroscopic materials. Relative hygroscopicity of the gelatin capsules and the filling materials determines the direction of vapor phase transfer of water in the closed capsule system. Gelatin capsules offer little protection to a hygroscopic content from atmospheric water vapor. Certain coatings may enhance this protective function, and the extent of protection may be evaluated in terms of prolonged diffusion lag time.

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Preparation and Measurement of Samples.—Stock samples of wetted calcium carbonate were prepared from calcium carbonate powder (A.R. grade) and distilled water. For each stock sample, 100 Gm. of calcium carbonate was mixed with the

calculated amount of water with a mechanical mixer. The samples were stored in screw-cap glass jars and the actual moisture contents were determined by gravimetric analysis using 2- to 3-Gm. samples dried to constant weight in weighing bottles at 100°.

Preliminary experimentation showed that the sample layer must be as uniformly dense as possible in order to get reproducible results. Slugs having convex surfaces were made from the stock samples with a 1-in. diameter punch and die set and a Carver laboratory press. The appropriate weight of sample, determined from a mass absorption curve, was added to the die, and a pressure of 5000 pounds was gradually applied to the sample. The moisture content of the slugs was limited to a range of 0.5–10%, since below 0.5% the powder was too dry to press into slugs and above 10% water droplets were pressed out of the powder. Each slug was placed in a geometrically reproducible position over the SrY-90 source under the Geiger-Müller tube in the lead shield. The distance from the top of the slug to the thin window of the tube was 1.8 cm. The beta-particle transmission was determined during 2 minutes.

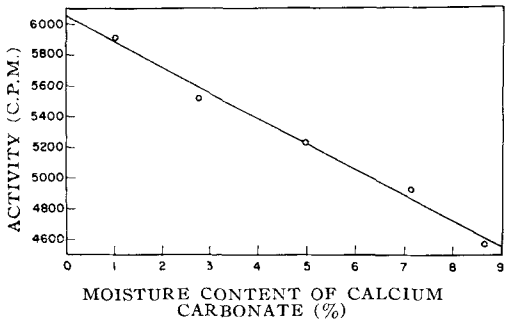


Fig. 1.—SrY-90 beta-particle transmission through wetted calcium carbonate using a constant dry sample weight.

RESULTS

Five stock samples with moisture contents ranging from 1.04 to 8.65% were studied using a constant dry-sample weight of 2.75 Gm. The beta-particle transmissions of four slugs from each stock sample were measured and the average transmission was calculated. The moisture contents and average transmissions of the five stock samples were: 1.04%, 5,913 ± 131 c.p.m.; 2.79%, 5,521 ± 73 c.p.m.; 4.97%, 5,229 ± 47 c.p.m.; 7.13%, 4,930 ± 61 c.p.m.; and 8.65%, 4,568 ± 72 c.p.m. Figure 1 is a least squares plot of these data.

Four stock samples with moisture contents ranging from 1.04 to 7.13% were studied using a constant wet-sample weight of 2.89 Gm. The beta-particle transmissions of four slugs from each stock sample were measured and the average transmission was calculated. The moisture contents and average transmissions of the four stock samples were: 1.04%, 4,857 ± 88 c.p.m.; 2.79%, 5,033 ± 88 c.p.m.; 4.97%, 5,162 ± 41 c.p.m.; 7.13%, 5,294 ± 41 c.p.m. Figure 2 is a least squares plot of these data.

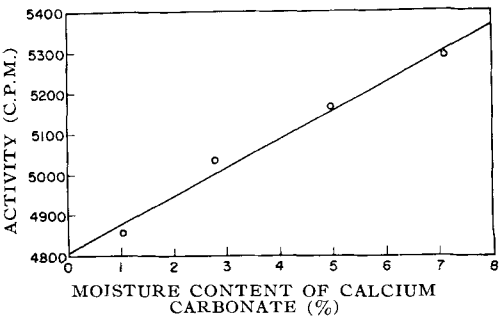


Fig. 2.—SrY-90 beta-particle transmission through wetted calcium carbonate using a constant wet sample weight.

DISCUSSION

The precision of the determinations could be improved by reducing the counting error. The standard deviations of the four determinations for each moisture content range from ±0.80 to ±2.21%. However, the expected counting error for the 2-min. counts is about 1%. The counting error could be reduced by either taking longer counts or using a more intense beta source. If a more intense beta source is used, a scintillation counter with an anthracene crystal would be desirable to avoid coincidence corrections.

The beta-particle transmission technique should be useful in routine moisture analyses of materials when moisture content is the only variable. Calibration curves relating beta-particle transmission to moisture content would be required for each material.

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Effects of Stimulants on Rotarod Performance of Mice

By N. PLOTNIKOFF†, D. REINKE, and J. FITZLOFF

This study has demonstrated that amphetamine and pheniprazine increased performance time of mice on the rotating rod, whereas the effects of caffeine were questionable.

SEVERAL reports in the literature have suggested that various stimulant drugs have differential effects on various motor coordination tasks. Studies in lower animals appeared to be limited and it seemed of interest to evaluate the effects of various stimulant drugs on motor coordination. The present study will attempt to describe the effects of a group of stimulants on the performance of mice on a rotating rod, a test originally reported by Dunham and Miya (1).

METHODS

The apparatus originally reported by Dunham and Miya (1) was modified in the following manner: the rod consisting of a 1-in. wooden dowel was divided into 10 equal spaces of 3.5 inches by plastic disks of 8 inches diameter. The rod is operated by a kymograph motor (Gorell and Gorell Monodrum) which has an adjustable speed. One end of the wooden dowel was fitted into the kymograph drive shaft and the other into a ball bearing (1 inch) supported by a vertical wooden support.

The actual testing procedure consisted of (*a*) training all animals by placing them on the rotarod for 6 minutes, (*b*) injecting the animals with drugs by the intraperitoneal route, and (*c*) 30 minutes following injection, observing the performance of the animals on the rotarod for 1 hour, or until they fell off.

RESULTS

Studies on control mice consisted of performance evaluation at three different rotarod speeds. The results are shown in Table I.

TABLE I.—PERFORMANCE TIME *vs.* SPEED

Rotarod Speed, r.p.m.	No. of Groups of Mice[a]	Average Performance Time ± S.E., min.
A 29.28	10	24.5 ± 3.0
B 18.30	5	28.9 ± 4.1
C 11.44	16	30.2 ± 2.2

[a] Ten mice per group.

Although the mean performance times of the mice at the three speeds did not appear to differ significantly, gross differences in behavior were noted. The animals tested at the A speed were observed to have difficulty keeping up with the speed of the rod while those tested at the C speed spent a great deal of time in exploratory behavior. Thus, mice dropping off the rotarod at the A speed may be said to do so from failure to "keep up" with the rod, while those tested at the C speed dropped off primarily because of errors in exploratory behavior—attempts to climb or lean against the plastic disk dividers and/or side excursions on the rod itself. The behavior of the mice tested at the B speed resembled those tested at the A speed.

The effects of amphetamine on performance time were evaluated on three different rotarod speeds, A, B, and C. The results are shown in Table II.

The data shown would indicate that there is a dose response relationship with the use of amphetamine at the three speeds. The estimated doses effective in enhancing performance in 50% of the animals (ED_{50}) were found to be 2.8 ± 0.4 mg./Kg. for the A speed, 1.9 ± 0.4 mg./Kg. for the B speed, and 5.1 ± 0.5 mg./Kg. for the C speed ("estimated" ED_{50} only, since upper ratio was 1.48 at 5.0 mg./Kg.). Thus, there is no apparent difference (by inspection of means and standard errors) between the effects of amphetamine on A and B speeds; however, the agent is less active at the C speed.

The effects of pheniprazine also were evaluated at the three speeds and the results are shown in Table III.

Significant increases in mean performance time with the use of pheniprazine were observed in all three speeds. However, at the C speed the agent was seemingly ineffective 30 minutes after administration (standard error limits compared to controls) but had a pronounced effect at 2 hours. The estimated ED_{50} doses are 6.0 ± 0.8 mg./Kg. for the A speed, 7.0 ± 1.3 mg./Kg. for the B speed, and 6.5 ± 0.8 mg./Kg. for the C speed.

Caffeine was also evaluated at the three speeds and the results are shown in Table IV.

There was no significant improvement in the performance of mice treated with caffeine at any speed tested.

DISCUSSION

The present investigation has demonstrated that the stimulants, amphetamine and pheniprazine, enhance the performance of mice on the rotarod, whereas caffeine was found to be inactive or to have a questionable effect.

It is debatable whether this enhancement is due to a decrease in errors of judgment in movement or an actual improvement in muscular coordination. Thus, control animals tested at the C speed exhibited exploratory behavior while those tested at the A and B speeds did so to a much smaller extent.

Following medication, the animals appeared to concentrate to a greater extent on maintaining pace with the rotating rod and spent less time in exploratory behavior. Several reports indicate that amphetamine reduces errors in the performance of

Received March 14, 1962, from the Stanford Research Institute, Menlo Park, Calif.
Accepted for publication April 6, 1962.
This work was supported by Contract Nonr 2993(00) between the Office of Naval Research and Stanford Research Institute.
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TABLE II.—EFFECT OF AMPHETAMINE ON PERFORMANCE

Speed	Dose, mg./Kg.	No. of Groups of Mice[a]	Mean Performance Time ± S.E., min.	Performance Ratios[b]
A	Controls	10	24.5 ± 3.0	1.00
	2.5	1	34.3 ± 7.3	1.40
	5.0	1	50.0 ± 1.0	2.04
	10.0	1	33.5 ± 8.0	1.36
B	Controls	5	28.9 ± 4.1	1.00
	1.25	1	37.0 ± 0.3	1.28
	2.5	1	51.3 ± 8.4	1.77
	5.0	1	52.5 ± 4.2	1.81
C	Controls	16	30.2 ± 2.2	1.00
	1.25	1.5	29.1 ± 9.4	0.96
	2.5	3.5	33.1 ± 2.5	1.09
	5.0	3.5	44.7 ± 4.9	1.48

[a] Ten mice per group. [b] Experimental/control.

TABLE III.—EFFECT OF PHENIPRAZINE ON PERFORMANCE

Speed	Pretreatment Time, hr.	Dose, mg./Kg.	No. of Groups	Mean Performance Time ± S.E., min.	Performance Ratios[a]
A	1/2	Controls	10	24.5 ± 3.0	1.00
		2.5	1	31.7 ± 16.7	1.29
		5.0	1	29.6 ± 12.7	1.20
		10.0	1	53.9 ● 6.2	2.19
B	1/2	Controls	5	28.9 ± 4.1	1.00
		2.5	1	29.4 ± 4.0	1.01
		5.0	1	43.1 ± 6.8	1.49
		10.0	1	44.6 ± 17.2	1.54
C	1/2	Controls	16	30.2 ± 2.2	1.00
		2.5	1	26.1 ± 12.7	0.86
		5.0	1	42.4 ± 10.0	1.40
		10.0	1	36.5 ± 8.7	1.20
C	2	Controls	16	30.2 ± 2.2	1.00
		2.5	0.5	37.7 ± 10.1	1.24
		5.0	1	43.3 ± 0.2	1.43
		10.0	2	47.2 ± 7.7	1.56
		20.0	0.5	60.0 ± 0.0	1.98

[a] Experimental/control.

TABLE IV.—EFFECT OF CAFFEINE ON PERFORMANCE

Speed	Dose, mg./Kg.	No. of Groups	Mean Performance Time ± S.E., min.	Performance Ratios[a]
A	Controls	10	24.5 ± 3.0	1.00
	5.0	1	12.9 ± 0.6	0.53
	10.0	1	18.2 ± 0.1	0.74
	20.0	1	6.6 ± 1.0	0.27
B	Controls	5	28.9 ± 4.1	1.00
	5.0	1	30.9 ± 4.2	1.07
	10.0	1	29.3 ± 7.3	1.01
	20.0	1	36.3 ± 1.6	1.27
C	Controls	16	30.2 ± 2.2	1.00
	5.0	1	30.1 ± 2.0	1.00
	10.0	1	26.3 ± 2.15	0.87
	20.0	1	32.5 ± 2.60	1.08

[a] Experimental/control.

repetitive tasks and improves muscular coordination in human subjects (2). Thus, this bioassay may be useful in characterizing the effects of other stimulants on motor coordination.

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Identification Test for Tolbutamide and Chlorpropamide

By BERTON E. BALLARD and EINO NELSON

To date no simple and rapid identification test for tolbutamide or chlorpropamide is available, although more elaborate qualitative and quantitative methods of detecting and determining the compounds have been proposed (1–12). The qualitative test presented here is based on a "spot" test discussed by Feigl (13) for the detection of methenamine.

PROCEDURE

To a 100-mg. sample of each of the drugs listed in Table I, 100 mg. of powdered zinc and 100 mg. of granular ammonium chloride were added and intimately mixed in a mortar. The powder mixture of each drug was transferred to an 8-in. Pyrex test tube. Test papers were made by dipping Whatman No. 5 filter paper into a 2% solution of 2,4-dinitrochlorobenzene dissolved in ethyl ether. The paper was dried, passed through the vapors of concentrated ammonium hydroxide, and cut into squares large enough to completely cover the test tube opening.

The identification test for the compounds consisted of placing the square of test paper over the tube opening and putting the tube in a Slaco tube heater (Hallikainen Instruments, Berkeley, Calif.) preset at 150 ± 1° for exactly 10 minutes. The paper was held tightly in place by resting a small Pyrex beaker on top of it during the heating period. A yellow circular stain on the part of the paper over the tube orifice at the end of the heating period indicated a positive result for a particular compound. The lower limits of the method for the detection of tolbutamide and chlorpropamide under these conditions were determined by keeping the weights of zinc powder and ammonium chloride constant and decreasing the quantity of added sulfonylurea until a definite yellow paper color was seen at the end of 35 minutes.

Tolbutamide, chlorpropamide, methenamine, and sulfisoxazole all gave color reactions on the paper within the 10-min. time period. The yellow color of the first three substances on paper was the one expected for the reaction between a volatile amine and 2,4-dinitrochlorobenzene, while the brown color seen with sulfisoxazole was atypical and due to the staining of the paper by colored distillation products.

The method could detect 0.4 mg. of either tolbutamide or chlorpropamide after 35 minutes of heating.

Table I.—Drugs to which Powdered Zinc and Granular Ammonium Chloride Were Added to Make Test

	Official or Common Name[a]	Results
1.	Aprobarbital	−
2.	Barbital	−
3.	5-*n*-Butyl-5-ethylthiobarbituric acid	−
4.	Chlorpropamide	+
5.	5,5-Diethylthiobarbituric acid	−
6.	Methenamine	+
7.	Pentobarbital	−
8.	Phenobarbital	−
9.	Sulfacetamide sodium	−
10.	Sulfaguanidine	−
11.	Sulfamethazine	−
12.	Sulfamethizole	−
13.	Sulfisoxazole	+[b]
14.	Thiopental	−
15.	Tolbutamide	+

[a] 1. 5-Allyl-5-isopropylbarbituric acid; 2. 5,5-diethylbarbituric acid; 4. 1-propyl-3-(*p*-chloro-benzenesulfonyl)-urea; 6. formin or hexamethylenetetramine; 7. 5-ethyl-5-(1-methylbutyl)barbituric acid; 8. 5-ethyl-5-phenylbarbituric acid; 9. N¹-acetylsulfanilamide sodium; 10. N¹-Guanylsulfanilamide; 11. N¹-(4,6-dimethyl-2-pyrimidinyl)sulfanilamide; 12. N¹-(5-methyl-1,3,4-thiadiazol-2-yl)sulfanilamide; 13. N¹-(3,4-dimethyl-5-isoxazolyl)-sulfanilamide; 14. 5-ethyl-5-(1-methylbutyl-)2-thiobarbituric acid; 15. 1-butyl-3-(*p*-tolylsulfonyl)urea. [b] Brown tint to paper due to volatile decomposition products.

DISCUSSION

The pyrolysis reaction here probably involves first the liberation of hydrogen chloride from the heated ammonium chloride-zinc dust mixture, which then causes cleavage of the substituted sulfonylurea. An amine is liberated as a decomposition product. If ammonium chloride is omitted from the procedure, the development of the yellow ring is greatly delayed. The identification test described here is also suitable for qualitative detection of the hypoglycemic agents in commercially available tablets.

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Received December 13, 1961, from the Department of Pharmacology and the School of Pharmacy, University of California Medical Center, San Francisco 22.
Accepted for publication January 15, 1962.
The assistance of Mr. Victor Wong is gratefully acknowledged. The tolbutamide and chlorpropamide used in this study were gifts from the Upjohn Co. and Chas. Pfizer and Co., respectively.

Colorimetric Determination of Salicylic Acid

By JOSEPH D. DeMARCO and ARNOLD D. MARCUS

DURING a study of the stability of various salicylic acid derivatives, it was necessary to follow the decomposition reactions by determining the free salicylic acid produced. Because of various interferences, direct ultraviolet determination appeared impractical. It seemed advisable, therefore, to attempt to make use of the well-known ferric ion-salicylic acid reaction.

Two recent reports concerning the stability of aspirin describe modifications of the A.O.A.C. procedure (1) for salicylic acid. These reports concern the use of ferric alum reagent in reaction systems containing 50% ethanol (2) and the same color developing material in aqueous solutions adjusted to pH 2.63 (3). Both modifications appeared unsatisfactory for our need because of the very low concentrations of salicylic acid used. The resulting low absorbance values, on an instrument such as the Beckman model DU spectrophotometer, appeared to allow too great a chance for inaccuracy and imprecision.

Since it seemed probable that the low concentrations of salicylic acid (1–2 mg./100 ml.) were used in an effort to maintain fidelity to Beer's law, we were interested in determining if the method could be improved to permit use of higher concentrations of salicylic acid. The following summarizes our studies concerning the effect of ethanol concentration and the relative usefulness of ferric chloride as compared to ferric alum.

EXPERIMENTAL

Reagents.—Salicylic acid U.S.P.; absolute ethanol, reagent; ferric chloride hexahydrate, Merck reagent; ferric alum, Merck reagent.

Procedure.—Standard solutions of salicylic acid were prepared using absolute ethanol as the solvent. Aliquots of these solutions were then transferred to 100-ml. volumetric flasks, the desired amounts of the ferric chloride or ferric alum solutions were added along with additional ethanol, when desired or necessary, and the solution brought to 100 ml. After 15 minutes the color was read at 532 mμ against a suitable reagent blank. All readings were made on a Beckman model DU spectrophotometer using the tungsten lamp.

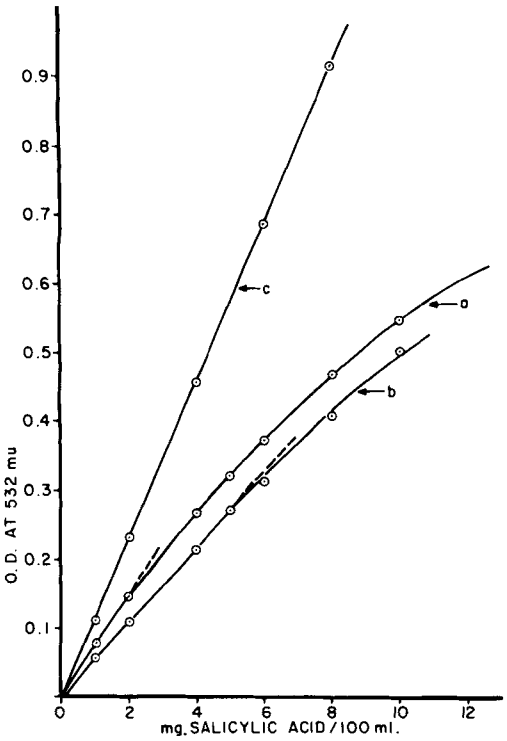


Fig. 1.—Plots showing the effect of ethanol and ferric alum concentration on the iron-salicylate reaction; 5 ml. ferric chloride (1%) in 50% ethanol system.

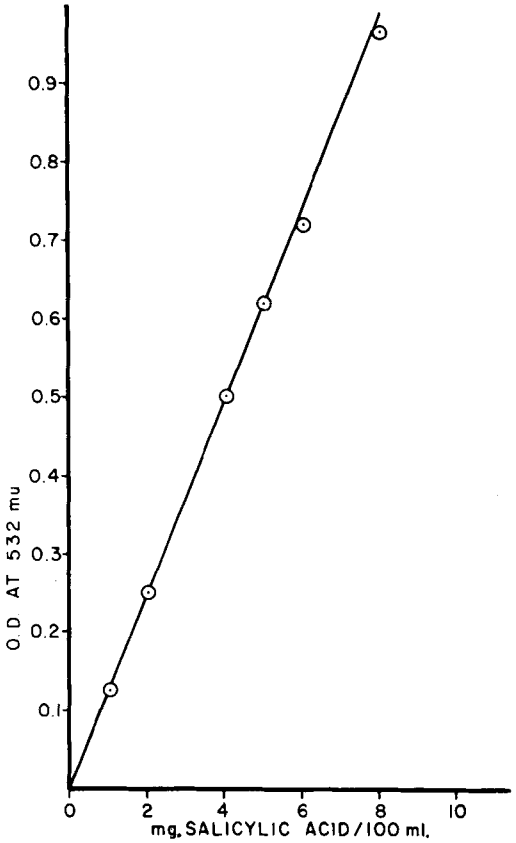


Fig. 2.—Plot showing the value of ferric chloride in gaining adherence to Beer's law despite a high ethanol concentration; 5 ml. ferric chloride (1%) in 50% ethanol system.

Received January 15, 1962, from the Pharmaceutical Research Department, Merck Sharp and Dohme Research Laboratories, West Point, Pa.
Accepted for publication February 15, 1962.

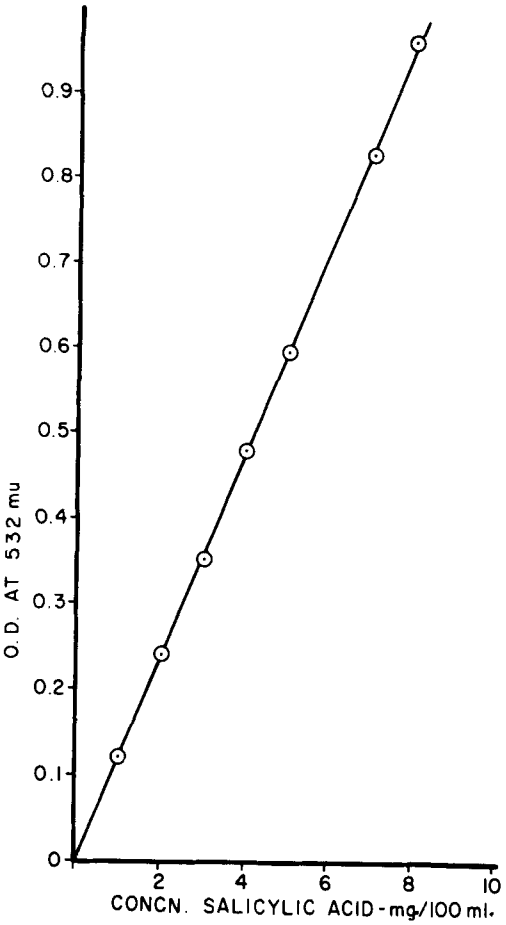


Fig. 3.—Plot showing ferric chloride to be only barely affected by ethanol concentration in the color reaction with salicylate (compare with Fig. 2); 5 ml. of 1% ferric chloride in 10% ethanol system.

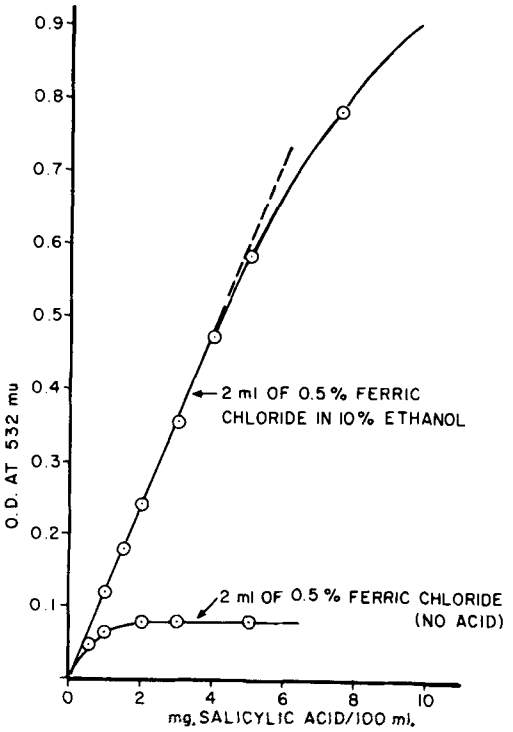


Fig. 4.—Plots showing the importance of suitably high concentrations of acid for good development of the iron-salicylate color.

DISCUSSION

As shown in Fig. 1, curve A, the use of 2 ml. of a 2% solution of ferric alum in a system containing 50% ethanol gives but limited adherence to Beer's law. Somewhat greater fidelity is achieved by increasing the amount of reagent added to 5 ml. per 100 ml. of reaction mixture (curve B). The best adherence to linearity is obtained, however, when the ferric alum reagent is maintained at 5 ml. and the ethanol concentration is reduced to 10% of the final volume (curve C). Indeed, this last modification provided not only good linearity but apparently increased the efficiency of the reagent considerably. By increasing the amount of ferric alum and reducing the alcohol concentration, sufficiently high concentrations of salicylic acid can be used so that absorbance readings in the region of maximum accuracy and precision are easily attainable.

Since some studies might require the use of sufficient ethanol to make the use of ferric alum undesirable, we studied the usefulness of ferric chloride to develop the characteristic color. This material is given as an alternate for ferric alum in the A.O.A.C. method (1). Solutions containing 0.5 and 1.0% ferric chloride in 0.1 *N* hydrochloric acid were used for color development.

As shown in Fig. 2, the use of 5 ml. of 1.0% ferric chloride in a system containing 50% ethanol provided good adherence to Beer's law. By reducing the ethanol concentration to 10%, linearity is extended to higher concentrations of salicylic acid. This is demonstrated in Fig. 3.

When the 1.0% solution of ferric chloride is replaced with a 0.5% solution, Beer's law is obeyed up to a concentration of 5 mg. of salicylic acid per 100 ml., as in Fig. 4. Although this is useful from a practical viewpoint, the use of a 1.0% solution of ferric chloride seems preferable.

The lower curve in Fig. 4, obtained with a 0.5% aqueous solution of ferric chloride (no added acid) convincingly demonstrates the necessity of an acid system for suitable color development.

CONCLUSION

On the basis of these studies, it may be concluded that ferric chloride is preferred to ferric alum as a reagent for the colorimetric determination of salicylic acid. If the latter must be used, the ethanol concentration should be kept to a minimum. In either case, preparation of the reagent in acid solution is mandatory for proper color development.

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Synthesis of Salicylamide Sulfate and its Effect on Calcium Ion Solubility

By WILLIAM O. FOYE, RONALD N. DUVALL, MONTGOMERY H. TALBOT, and ROBERT H. ZAIM

Salicylamide sulfate has been synthesized and found to be chromatographically identical to a metabolic product appearing in the urine of patients on salicylamide therapy. This compound, when incubated with suspensions of calcium hydroxide, was found to increase the aqueous solubility of calcium ion beyond that expected from a salt effect alone. An explanation of the effect of salicylamide in halting calcium stone growth has been suggested.

SALICYLAMIDE SULFATE (I), commonly referred to as an "ethereal" sulfate but actually a sulfate ester, has been postulated as a metabolite of salicylamide in humans. Litter, *et al.* (1), claimed that 36% of the drug was excreted by humans as the sulfate without giving experimental details, and Becher, *et al.* (2), showed that a metabolite of salicylamide from humans liberated sulfate after hydrolysis. Foye, *et al.* (3), also hydrolyzed the urines collected from stone-formers and found that a metabolite, supposed to be the sulfate according to Becher's procedure, liberated both salicylamide and sulfate in the hydrolysate. Quantitative determinations on the urines of a large number of stone-forming patients gave an average "ethereal" sulfate excretion of 31% of the salicylamide ingested. Mandel, *et al.* (4), did not detect the sulfate among the metabolites of salicylamide from three cancer patients, but possibly their countercurrent extraction procedure was too lengthy to obtain the sulfate unhydrolyzed.

Salicylamide sulfate has not previously been isolated. To identify positively that the sulfate measured quantitatively in human urines was salicylamide sulfate, the compound has now been synthesized and shown to be chromatographically identical with the urinary product. Although the substance did not migrate with the solvent systems used, the faintly flourescent spot it gave on paper chromatograms did not appear in the urines of the patients in the absence of salicylamide ingestion. A supply of this compound was also required to determine the extent of any solubilization it might exert on calcium ion, since salicylamide ingestion has been found to halt calcium stone growth in a number of stone-formers (5).

The procedure of Burkhardt (6) for preparing the sulfate of phenol using dimethylaniline and potassium pyrosulfate was attempted for the synthesis of salicylamide sulfate, but the major product was dimethylaniline-*m*-sulfonic acid. Baumann's procedure (7) for the preparation of salicylic acid sulfate (no per cent yield was mentioned), however, was adapted to the present synthesis, and the potassium salt of salicylamide sulfate was obtained in low yield. The low yield is not surprising when it is considered that the compound was prepared in an aqueous medium and is readily hydrolyzable. The compound was found to be quite hygroscopic and unstable when exposed to air for any length of time, which probably accounts for the discrepancy in the analytical value for carbon. The product was chromatographically homogeneous, however, and showed no evidence of contamination with salicylamide or other salicylic acid derivatives. Attempts to prepare salicylamide sulfate under nonaqueous conditions were unsuccessful.

Solubility determinations were made in water for calcium hydroxide in the presence of salicylamide sulfate, as well as with sodium chloride, measurements being made up to 3 hours, when hydrolysis of the salicylamide sulfate becomes noticeable. A definite increase in the solubility of calcium hydroxide was noted beyond that to be expected from the salt effect alone (see Table I). Examination of the data in Table I reveals an apparent increase with time in the solubility of calcium hydroxide when in the presence of salicylamide sulfate. This could be due to slow formation of a calcium ion salicylamide sulfate complex, but is more likely the result of experimental variation or error. The same may be said for the apparent decrease in solubility of calcium hydroxide in water or in the presence of sodium chloride.

It appears likely that a so-called weak salt or complex is formed between salicylamide sulfate and

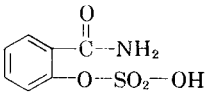
TABLE I.—SOLUBILITY OF CALCIUM ION IN THE PRESENCE OF SALICYLAMIDE SULFATE AND AN INDIFFERENT SALT

Sample	Substance Added	Time, min.	$[Ca^{++}]$, m*M*/ml.
1	0.04 *M* SS[a]	40	0.0410
2	0.04 *M* SS[a]	105	0.0411
2	0.04 *M* SS[a]	145	0.0423
4	0.04 *M* SS[a]	185	0.0433
1	0.04 *M* NaCl	40	0.0287
2	0.04 *M* NaCl	105	0.0263
3	0.04 *M* NaCl	145	0.0240
4	0.04 *M* NaCl	185	0.0266
1	0.03 *M* SS	40	0.0262
2	0.03 *M* SS	105	0.0276
3	0.03 *M* SS	145	0.0292
4	0.03 *M* SS	185	0.0291
1	0.03 *M* NaCl	40	0.0204
2	0.03 *M* NaCl	105	0.0190
3	0.03 *M* NaCl	145	0.0177
4	0.03 *M* NaCl	185	0.0197
1	none	40	0.0186
2	none	105	0.0182
3	none	145	0.0168
4	none	185	0.0160

[a] SS, salicylamide sulfate.

Received November 16, 1961, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.
Accepted for publication January 29, 1962.
Supported in part by a grant (A-1914) awarded by the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

calcium ion, which could explain the halt in calcium stone growth observed in stone-formers when taking salicylamide daily. While the increase in solubilization is small [molar concentrations of salicylamide sulfate were used which approximate those to be expected in urines from the amount of salicylamide (2–3.6 Gm.) ingested by stone-formers on this therapy], it is sufficient to explain the failure of existing stones to accumulate the few mg. of calcium salts per day that ordinarily deposit in the kidneys of stone-forming patients.



I

EXPERIMENTAL

Potassium Salicylamide Sulfate.—Salicylamide (20 Gm., 0.146 mole) was dissolved in 40 ml. of distilled water containing 16 Gm. (0.285 mole) of potassium hydroxide. Finely powdered potassium pyrosulfate (34 Gm., 0.134 mole), previously heated to 350–400°, was added slowly, with stirring. The mixture was stirred for 6 hours at a temperature of 60–70°, and the pH was maintained between 8 and 9 by the addition of saturated potassium hydroxide solution.

The mixture was then shaken with 50 ml. of 95% ethanol and filtered. After cooling, the mixture was treated with 150–200 ml. of ether, with shaking. The aqueous layer was separated and neutralized to pH 7 by the dropwise addition of glacial acetic acid. Precipitated salicylamide was removed, and the filtrate was evaporated to dryness by means of a current of dry air. Absolute ethanol (200 ml.) was added to the residue, and a white, powdery product was filtered, washed with absolute ethanol, and air-dried for a few hours before being stored in a desiccator. The yield was 1.42 Gm. of product which softened at 145–150° and melted at 220–230°.

Anal.[1]—Calcd. for $C_7H_6KNO_5S$: C, 32.93; H, 2.37; S, 12.56. Found: C, 31.70; H, 2.35; S, 12.76.

The compound is extremely soluble in water and insoluble in absolute ethanol. Its aqueous solution has a nearly neutral pH and gave a violet color with ferric chloride T.S. only after hydrolysis with dilute hydrochloric acid. It gave no precipitate with barium chloride solution and dilute hydrochloric acid until the solution was heated for a few minutes. The barium sulfate was isolated, weighed, and the percentage of sulfur calculated. The total sulfur present was thus shown to be sulfate ester.

The compound gave a positive flame test for potassium. It is unstable in air at room temperature. An aqueous solution showed the same faintly fluorescent spot on a paper chromatogram, which did not migrate in the solvent system used, 1-butanol saturated with 3 *N* ammonia, as was obtained in the urines of patients on salicylamide therapy, but not in the absence of salicylamide ingestion. Hydrolysis of the material liberated salicylamide and sulfate ion.

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Digitoxin Antagonism by Visnadin

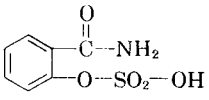
By D. D. VARONOS, P. C. VOUKYDES, and G. C. NIKITOPOULOU

Visnadin, a lactone derived from bishop's weed, has been found to have marked antidotal properties in animals poisoned with digitoxin. It increases survival on acute and chronic administration of the glycoside, prevents the appearance of bradycardia, and reverses cardiac arrhythmias.

DIGITALIS glycosides probably have the smallest margin of safety of any of our commonly used drugs and they appear to have resisted all attempts at improvement in this respect by chemical modification. A less desirable alternative would be to have available a reliable antidote, but this has not yet been found. Recently, a naturally occurring lactone, visnadin (1), has been introduced in Europe for the treatment of the anginal syndrome (2) and has been shown by Smith, *et al.* (1), to have 10 times

Received March 2, 1962 from the Department of Physiology, Medical School, University of Athens, Athens, Greece.
Accepted for publication March 20, 1962.

calcium ion, which could explain the halt in calcium stone growth observed in stone-formers when taking salicylamide daily. While the increase in solubilization is small [molar concentrations of salicylamide sulfate were used which approximate those to be expected in urines from the amount of salicylamide (2–3.6 Gm.) ingested by stone-formers on this therapy], it is sufficient to explain the failure of existing stones to accumulate the few mg. of calcium salts per day that ordinarily deposit in the kidneys of stone-forming patients.



I

EXPERIMENTAL

Potassium Salicylamide Sulfate.—Salicylamide (20 Gm., 0.146 mole) was dissolved in 40 ml. of distilled water containing 16 Gm. (0.285 mole) of potassium hydroxide. Finely powdered potassium pyrosulfate (34 Gm., 0.134 mole), previously heated to 350–400°, was added slowly, with stirring. The mixture was stirred for 6 hours at a temperature of 60–70°, and the pH was maintained between 8 and 9 by the addition of saturated potassium hydroxide solution.

The mixture was then shaken with 50 ml. of 95% ethanol and filtered. After cooling, the mixture was treated with 150–200 ml. of ether, with shaking. The aqueous layer was separated and neutralized to pH 7 by the dropwise addition of glacial acetic acid. Precipitated salicylamide was removed, and the filtrate was evaporated to dryness by means of a current of dry air. Absolute ethanol (200 ml.) was added to the residue, and a white, powdery product was filtered, washed with absolute ethanol, and air-dried for a few hours before being stored in a desiccator. The yield was 1.42 Gm. of product which softened at 145–150° and melted at 220–230°.

Anal.[1]—Calcd. for $C_7H_6KNO_5S$: C, 32.93; H, 2.37; S, 12.56. Found: C, 31.70; H, 2.35; S, 12.76.

The compound is extremely soluble in water and insoluble in absolute ethanol. Its aqueous solution has a nearly neutral pH and gave a violet color with ferric chloride T.S. only after hydrolysis with dilute hydrochloric acid. It gave no precipitate with barium chloride solution and dilute hydrochloric acid until the solution was heated for a few minutes. The barium sulfate was isolated, weighed, and the percentage of sulfur calculated. The total sulfur present was thus shown to be sulfate ester.

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Visnadin, a lactone derived from bishop's weed, has been found to have marked antidotal properties in animals poisoned with digitoxin. It increases survival on acute and chronic administration of the glycoside, prevents the appearance of bradycardia, and reverses cardiac arrhythmias.

DIGITALIS glycosides probably have the smallest margin of safety of any of our commonly used drugs and they appear to have resisted all attempts at improvement in this respect by chemical modification. A less desirable alternative would be to have available a reliable antidote, but this has not yet been found. Recently, a naturally occurring lactone, visnadin (1), has been introduced in Europe for the treatment of the anginal syndrome (2) and has been shown by Smith, *et al.* (1), to have 10 times

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the activity of khellin and four times that of papaverine as a coronary vasodilator (2). Since this drug will inevitably be used in digitalized patients, it seemed desirable to study its pharmacologic relationship to the glycosides.

METHODS AND RESULTS

The effect of visnadin was studied first on the acute LD_{50} of digitoxin in adult male, albino mice. Digitoxin was administered by subcutaneous injection using 10 animals for each dose level. The LD_{50} was calculated by the method of Miller and Tainter (3). Two mice of approximately equal weight were assigned to each cage. After several hours fasting, they were given a mixture of one-half of their usual daily food intake mixed intimately with the appropriate dose of visnadin. In this way, the dose was entirely consumed. Fresh food was given 12 hours later and any unconsumed portion removed from the cages after 4 hours. Finally the digitoxin was injected after another 4 hours. The results are given in Table I from which it appears that visnadin at the maximum feasible oral dose raised the LD_{50} of digitoxin approximately 70%.

TABLE I.—EFFECT OF VISNADIN ON THE LETHAL DOSE OF DIGITOXIN IN THE MOUSE

Dose of Visnadin, mg./Kg.	LD_{50} of Digitoxin ± S.E., mg./Kg.
0	14.4 ± 0.48
150	18.2 ± 0.79
300	24.4 ± 0.78
ca. 450[a]	24.2 ± 1.03

[a] Food-drug mixture not entirely consumed.

Visnadin was also found to modify the lethal dose of digitoxin on chronic administration in the mouse. Digitoxin was given subcutaneously at a dose of 2.5 mg./Kg. daily for 2 days and then 3.5 mg./Kg. daily until death occurred. One group of 10 mice received digitoxin only, while a second group of 10 were given the same dose of digitoxin and, simultaneously, 400 mg./Kg. of visnadin in the food as in the preceding experiments. The control group survived for an average of 9.3 days and the visnadin-treated group for an average of 12.8 days. The mean lethal doses were 25 and 35 mg./Kg. for control and treated group, respectively.

Digitoxin was administered daily by subcutaneous injection to adult albino rats with and without the addition of visnadin to the feed by a technique similar to that described above. Electrocardiograms were taken daily before the dose of digitoxin was given. The duration of the cardiac cycle was measured and the data are given in Table II. It is clear that visnadin strongly inhibited the development of bradycardia due to digitoxin.

DISCUSSION

The mechanism of the antagonistic action of visnadin on the toxicity of the cardiac glycosides is not known. It may possibly be related to its coronary vasodilator action since Lee (4) and Reiter (5) have reported that the positive inotropic action of the glycosides is followed by a deficiency of oxygen in the heart muscle. However, both visnadin and the digitalis glycosides contain unsaturated lactone rings and have certain other structural similarities, so that competitive antagonism must also be considered. Several unrelated lactones have been reported to have antidigitalis glycoside activity (6, 7), although results of other workers do not absolutely agree with this view (8). Nevertheless, it must be considered that the lactones of the cardiac glycosides, except scillaren, are furane forms, while the visnadin lactone belongs to the pyrane form.

It seems clear that visnadin merits further study as to mechanism of action and clinical application because of its great potential value in controlling the action of the digitalis glycosides in the treatment of heart disease.

TABLE II.—EFFECT OF VISNADIN ON BRADYCARDIA PRODUCED BY DIGITOXIN IN THE RAT

Time	Cardiac Cycle Duration, sec., + mean of 10 animals ± S.E. Group I Digitoxin, 2.5 mg./Kg. per day, s.c.	Group II Digitoxin, *ibid.*, plus Visnadin, 200 mg./Kg. per day, oral	Probability of Difference between Means Due to Random Sampling[a]
Control	0.138 ± 0.0040	0.139 ± 0.0039	0.8
Digitoxin started			
1st Day	0.170 ± 0.0039	0.168 ± 0.0037	0.8
Visnadin started			
2nd Day	0.183 ± 0.0032	0.158 ± 0.0039	0.001
3rd Day	0.198 ± 0.0035	0.149 ± 0.0046	0.001
4th Day	0.210 ± 0.0049	0.147 ± 0.0048	0.001
5th Day	Death	0.160 ± 0.0041	

[a] Based on Fischer's *t* test.

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Communications

Riboflavin Marker in Drug Products

Sir:

In patients receiving long-term therapy, a simple procedure is desirable for determining whether or not the patients are actually taking their prescribed medication. This might be done by including, in the dosage form, a marker which is easily detectable in the urine. Riboflavin has been suggested for this purpose by several investigators. This communication reports a brief examination of the use of riboflavin in this manner.

Control urine samples were collected from three human subjects on uncontrolled diets. Three milligrams of riboflavin was then administered to each subject. Urine samples were again collected after 3 hours.

Two subjects collected control urine samples prior to receiving orally, on different days, 3 mg. riboflavin plus one of the following drugs: 10 mg. chlorpromazine, 5 mg. prochlorperazine, 1 mg. trifluoperazine, 10 mg. thioridazine, and 5 mg. tranylcypromine. Urine samples were collected 3 hours after the subject had received the drug plus riboflavin.

The urine samples were observed in transparent flint glass bottles under the following conditions: In a darkroom, the urine samples were illuminated with a Black-Ray Model B-100 long-wave ultraviolet lamp.[1] The control urine samples exhibited a light blue fluorescence. The other urine samples exhibited a milky white fluorescence with distinct yellow at the sides of the bottle. Riboflavin in water shows a very intense yellow fluorescence under these conditions.

The control and test urine samples did not look appreciably different under normal illumination; however, under long-wave ultraviolet light they were easily distinguishable. Urine samples from subjects receiving riboflavin alone or riboflavin plus drug were indistinguishable.

For subjects receiving these, and possibly other drugs, three or four times daily, this amount of a riboflavin marker per dose seems more than sufficient to aid in establishing that the prescribed medication is actually being taken by simple periodic urine checks.

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Received August 9, 1962.
Accepted for publication August 21, 1962.

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The Ciba Collection of Medical Illustrations. Vol. 3, Digestive System. Part 2, Lower Digestive Tract. Prepared by FRANK H. NETTER. Ciba Pharmaceutical Co., Summit, N. J., 1962. xi + 243 pp. 24 × 30.5 cm. Price $15.

The second part of volume 3 in this exceptional series of informative art and comment includes seven sections under the headings: Development of the digestive tract, Anatomy of the abdomen, Anatomy of the lower digestive tract, Functional and diagnostic aspects of the lower digestive tract, Diseases of the lower digestive tract, Diseases and injuries of the abdominal cavity, and Hernias. References to the text in the sections (VIII–XIV) and a general subject index are appended.

Steroid Drugs. By NORMAN APPLEZWEIG. The Blakiston Division, McGraw-Hill Book Company, Inc., 330 West 42nd St., New York 36, N. Y., 1962. xv + 742 pp. 15 × 22.5 cm. Price $25.

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JOURNAL OF

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF **Pharmaceutical Sciences**

November 1962 volume 51, number 11

Review Article

Gas Chromatography and Its Application to Pharmaceutical Analysis

By E. BROCHMANN-HANSSEN

THE INTRODUCTION of chromatography as an analytical tool is generally attributed to Tswett (1) who coined the term chromatographic adsorption analysis. In principle, there appears to be no restriction on the nature of the mobile and the stationary phases in chromatography, nor on the types of equilibria involved. This was first recognized by Martin and co-workers (2, 3) who used a liquid adsorbed on a solid support as the stationary phase. The solid support might be a finely powdered inert material, such as diatomaceous earth, starch, or glass beads, or it might be sheets or strips of filter paper. In either case, separation took place by a partitioning of the solutes between two immiscible phases. In their first report on the partition chromatographic method, published in 1941, Martin and Synge (2) pointed out that the moving phase need not be a liquid, but might just as well be a gas. In fact, they even discussed the advantages that such a system might have. At the time nobody seemed to realize the tremendous significance of this suggestion, and the idea lay dormant for about 10 years until Martin, this time in cooperation with James, proceeded to work out the experimental technique. Their first paper on gas-liquid chromatography was published in 1952 (4). Actually, the principle of gas chromatography had been used for a long time for the separation of gases by means of a solid adsorbant (5, 6). However, it was the introduction of gas-liquid chromatography by James and Martin which led to the widespread acceptance of gas chromatography in general as an analytical technique. Gas-solid chromatography has remained the standard method for separation of permanent gases and low molecular weight hydrocarbons, while gas-liquid chromatography has become the method of choice for separation, identification, and quantitative determination of a wide variety of complex mixtures. The transition from gas-solid to gas-liquid chromatography is often gradual (7, 8) and, with the low concentrations of liquid phases now commonly used for separation of high boiling compounds, adsorption phenomena and the tailing which results are often difficult to eliminate.

The main advantages of gas chromatography over other modifications of chromatography, in fact over all other separation procedures, are speed, sensitivity, and versatility. The low viscosity of the mobile phase permits a high rate of flow and a fast equilibration between the thin film of liquid and the gas. As a consequence, separation can become very rapid. In many instances half a dozen or more substances may be separated in a matter of a few seconds. The sensitivity of the new ionization detectors is of the order of 10^{-12} Gm. per ml. of gas; 0.01 mcg. or even less can often be detected. A wide variety of stationary liquid phases, from nonpolar to very polar materials, are available for separation of a multitude of substances. Although essentially an analytical technique, gram quantities of very pure

Received from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Supported in part by Grant M-3487 from the National Institutes of Health, Bethesda, Md.

materials can be isolated from high capacity columns.

LITERATURE

In spite of its recent origin, the literature on gas chromatography is already very extensive. More than 1600 papers were published in 1961 alone (9). In addition to the well-known abstracting periodicals dealing with chemical and related literature, two abstracting services devoted exclusively to gas chromatography are available (10, 11). Numerous review articles (9, 12–23), monographs (24, 25), and books (26–31) have been published. Papers presented at national and international symposia on gas chromatography have been collected and published in book form. The purpose of the present review is to present some of the fundamental aspects of gas chromatography in general and to deal in somewhat greater detail with its analytical applications with special emphasis on pharmaceutical analysis.

THEORETICAL PRINCIPLES

In gas-liquid chromatography, one is mainly concerned with the interactions between the solutes and the stationary liquid. There is only one liquid solution to be considered rather than two, as in liquid-liquid chromatography. This relative simplicity, coupled with the speed and reproducibility of gas chromatographic separations, has greatly stimulated an interest in the theoretical aspects of chromatography. Two approaches have been used to explain the various phenomena affecting retention, efficiency, and resolution. The "plate theory," first introduced by Martin and Synge (2) for liquid-liquid partition chromatography and developed further by Glueckauf (32), treats the chromatographic column as a discontinuous system analogous to a distillation column, involving a series of equilibria or theoretical plates. The "rate theory" (33–35) regards the chromatographic column as a continuous medium in which diffusion phenomena and mass transfer are taken into account. A thorough discussion of the theory of gas chromatography is beyond the scope of this review. The reader is referred to the original literature and to the several books and review articles on the subject.

Retention.—When vapors from a sample enter a gas chromatographic column they are retarded by the stationary phase and travel more slowly than the carrier gas itself. The rate at which each solute travels through the column depends, among other things, on its partition coefficient between the stationary liquid and the gas or, in other words, upon its vapor pressure above its solution in the liquid. The volume of gas passing through the column from the time of application of the sample to the emergence of the peak maximum is called the retention volume, V_R

$$V_R = t_R F_c \qquad \text{(Eq. 1)}$$

where t_R is the retention time measured from the time of sample injection to the appearance of the peak maximum, and F_c is the flow rate of the carrier gas measured at the outlet pressure and at the temperature of the column. The compressibility of the gas causes its linear velocity to increase along the length of the column due to a drop in pressure from the inlet to the outlet. The corrected retention volume, V_R^o, is calculated by introducing a correction factor, f, for the pressure drop in the column (4)

$$V_R^o = f V_R = f\, t_R F_c \qquad \text{(Eq. 2)}$$

where $f = {}^3/_2[(p_i/p_o)^2-1]/[(p_i/p_o)^3-1]$, p_i and p_o being the carrier gas pressure at the inlet and the outlet, respectively. Correction should also be made for the "dead volume" or "gas holdup" of the column, V_G^o. V_G^o may be estimated (36, 37) from the retention time of homologous compounds or measured as the corrected retention volume of a gas which will not be adsorbed by the column liquid, such as air

$$V_N = V_R^o - V_G^o = f\, t\, F_c \qquad \text{(Eq. 3)}$$

where V_N is the net retention volume, corrected for the pressure drop of the column, and t is the net retention time measured as the time between the air peak and the peak for the component being analyzed.

A fundamental equation relating the corrected retention volume to the partition coefficient and the amount of stationary liquid in the column is given by

$$V_R^o = V_G^o + K V_L = V_G^o + Kw/\rho \qquad \text{(Eq. 4)}$$

where K is the partition coefficient, V_L is the volume and ρ the density of the stationary liquid, both at the column temperature, and w is the weight of the liquid phase in the column. In order to obtain an expression for the retention volume that would be independent of the amount of liquid phase, Littlewood, Phillips, and Price (38) introduced the symbol, V_g, usually referred to as the specific retention volume. It is the net retention volume per gram of liquid phase reduced to standard temperature and pressure

$$V_g = 273\, V_N/Tw = 273\, K/T\rho \qquad \text{(Eq. 5)}$$

where T is the column temperature in °K.

Equation 4, relating the corrected retention volume to the product of the partition coefficient and the volume of stationary liquid, does not account for the changes in elution sequence which sometimes occur with changes in the concentration of liquid phase on the column. Martin (39) suggested that such changes, which are especially predominant with highly polar liquid phases, are due to adsorption of the solute on the surface of the liquid. He has modified Eq. 4 to include this phenomenon.

Column Efficiency.—The efficiency of a gas chromatographic column may, in the same way as a distillation column, be described in terms of its number of theoretical plates and calculated from the chromatogram by the expression

$$n = 16\,(t_R/b)^2 \qquad \text{(Eq. 6)}$$

where b is the base (in the same units as t_R) of the triangle formed by the tangents through the inflection points of the peak. The length of the column required to establish the equivalent of one equilibration between the gas phase and the stationary liquid is called "the height equivalent to a theoretical plate" (HETP). This value is obtained from the total number of plates (n) and the length of the column (l)

$$\text{HETP} = l/n \qquad \text{(Eq. 7)}$$

In linear, nonideal chromatography, such as gas-liquid chromatography, and in the absence of adsorptive effects, the peaks are symmetrical or nearly so, approximating a Gaussian distribution curve. However, the bands tend to broaden as they move down the column and the longer the retention time of a solute, the wider the emerging peak. This band broadening which reduces the efficiency of the column is due to several factors involving diffusion phenomena and resistance to mass transfer. Van Deemter, Zuiderweg, and Klinkenberg (34) have expressed the relationships as follows

$$\text{HETP} = A + B/u + Cu \qquad \text{(Eq. 8)}$$

or more fully

$$\text{HETP} = 2\lambda d_p + \frac{2\gamma D_{\text{gas}}}{u} + \frac{8}{\pi^2} \cdot \frac{K'}{(1+K')^2} \cdot \frac{d_f^2 u}{D_{\text{liq}}} \qquad \text{(Eq. 9)}$$

where λ is the statistical irregularity of the packing, d_p is the particle diameter of the support, D_{gas} and D_{liq} denote the molecular diffusion in the gas and the liquid phases, respectively, u is the average linear gas velocity of the carrier gas, γ is the labyrinth factor or tortuosity constant of the paths in the column, K' represents the product of the partition coefficient, K, and the ratio of the liquid volume to the gas volume of the column, and d_f is the mean diameter of the liquid film on the support.

The term A, referred to as "eddy diffusion," is due to the irregularities of stream paths in a porous medium. Van Deemter, *et al.* (34), found it to be independent of flow rate. It can be reduced by careful packing and by using uniform particle size of small diameter (40, 41). B represents molecular diffusion which is inversely proportional to the gas velocity, and C is concerned with resistance to mass transfer, expressing the lack of equilibration between the two phases. The effects of these factors are illustrated in Fig. 1 in which HETP is plotted against the average linear velocity of the carrier gas. The van Deemter equation has been widely discussed and there is general agreement that it presents a reasonably good picture of the conditions responsible for the spreading of chromatographic zones. However, recent work by several investigators has indicated a need for an additional term or terms similar to the C term, dealing with resistance to mass transfer in the gas phase (42, 43). The A term has also been the subject of much speculation and controversy (44, 45).

As expressed in the van Deemter equation and by several investigators, the apparent column efficiency is affected by the nature, flow rate, and pressure gradient (46, 47) of the carrier gas, the particle size of the support and the density of the

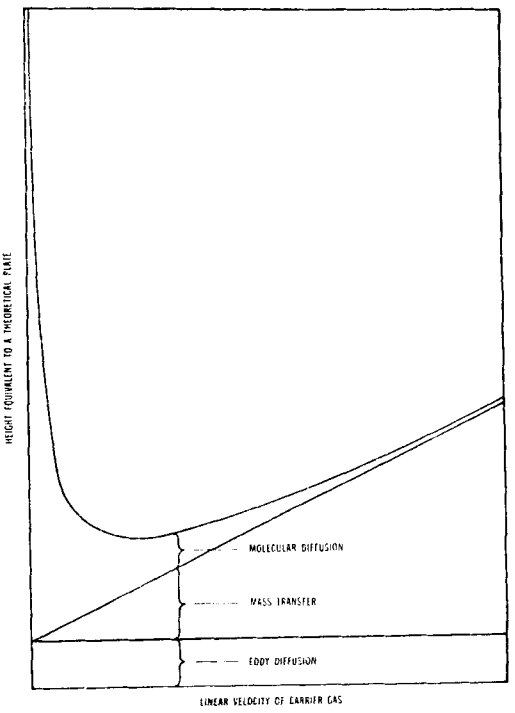


Fig. 1.—Effect of gas velocity on HETP. [After T. Johns (258); courtesy of Beckman Instruments.]

packing, the amount and type of liquid phase (49–51), the size and type of sample (40, 48), the column temperature (49, 50), the length of the column, and the conditions of sample introduction (51, 52). The response time and volume of the detection system are also important considerations (53, 54). Most packed gas chromatographic columns in use today have efficiencies of the order of 500 to 1000 theoretical plates per foot although much greater efficiency can be attained (41, 55). Golay (56) has suggested a "performance index" as a measure of column efficiency and has calculated on a theoretical basis the performance index for an ideal gas chromatographic column. From the difference between this theoretical value and those found in practice, he concludes that there is much room for improvement in basic column design.

Selectivity.—The relative position of the peaks in a gas chromatogram depends upon the relative vapor pressures of the solutes above the liquid phase in the column. The separation of two components in a mixture may, therefore, be expressed as the ratio of the partition coefficients or of the net retention volumes

$$\alpha_{1,2} = \frac{K_2}{K_1} = \frac{\gamma_1^o p_1^o}{\gamma_2^o p_2^o} = \frac{V_{N2}}{V_{N1}} \qquad \text{(Eq. 10)}$$

where α is the relative volatility of substances 1 and 2, γ_1^o and γ_2^o are the activity coefficients, of the solutes in the solvent at infinite dilution, and p_1^o and p_2^o are the vapor pressures of the pure substances.

Where there is little interaction between the solutes and the liquid phase, or where the solute-solvent interactions are of the same magnitude as the interaction between the solute molecules, gas chromatographic separation takes place largely according to the boiling points of the pure substances (57). Such stationary phases are nonselective. Nonpolar liquids, such as saturated hydrocarbons and alkylsilicones, belong to this group. Selectivity increases rapidly with increasing polarity due to the various cohesive forces operating between the solute and the liquid phase. A selective phase is one which will cause separation of substances which have the same boiling points but are of different molecular types (58–60). If $p_1^o = p_2^o$, Eq. 10 becomes

$$\alpha_{1,2} = \frac{\gamma_1^o}{\gamma_2^o} \qquad \text{(Eq. 11)}$$

This ratio has been referred to as the selectivity coefficient (58, 59). Hydrogen bonding plays a very important role in the solute-solvent interaction on polar liquid phases. This was clearly shown by James (61) in the separation of primary, secondary, and tertiary amines. On a nonpolar stationary liquid, the amines emerged in the order of increasing molecular weights, namely in the order of primary, secondary, and tertiary amines. On a very polar column the sequence was reversed, which is the order of increasing hydrogen bonding.

Permanent or induced dipoles in the solute will give rise to interaction with polarizable groups in the solvent and can be used for separation on selective stationary liquids (57). Formation of weak complexes has also been exploited as a means of increasing selectivity (62).

Resolution.—Resolution of closely related compounds in a mixture depends both on the efficiency of the column and on the relative volatility of the solutes in the stationary liquid. The relationship between column efficiency and resolving power has been studied by many workers (32, 55, 63–66).

An important parameter for effective separation is the ratio between the dead volume of the column, V_G^o, and the net retention volume of the solute, V_N. With a reduction in the amount of stationary phase on the column, the plate number often increases while the degree of separation decreases for components with a given relative volatility. This is, at least partly, compensated for by the lower temperature required with lightly loaded columns resulting in increased relative volatility (67). Purnell (55, 63) has defined a separation factor, S, which measures the ability of a chromatographic column to achieve a particular separation

$$S = 36\,[\alpha/(\alpha - 1)]^2 \qquad \text{(Eq. 12)}$$

From S the number of theoretical plates required for separation of two components, for which the relative volatility is known, can be calculated

$$n = 36\,[\alpha/(\alpha - 1)]^2[1 + 2\,(V_G^o/V_N) + (V_G^o/V_N)^2] \qquad \text{(Eq. 13)}$$

Purnell (55) showed that compounds with an α value of 1.15 will be completely separated on a column having 1500 theoretical plates if V_G^o is negligible, whereas, 40,000 plates are required if the ratio V_G^o/V_N is 10.

RECENT ADVANCES IN INSTRUMENTATION AND TECHNIQUES

Detectors.—There has been considerable development in the field of detectors for gas chromatography since James and Martin (4) analyzed eluted acids by a titration procedure. Although the workhorse among detectors for many years has been the katharometer, this de-

tector cell does not possess the sensitivity necessary for the small sample sizes commonly used with high molecular weight compounds and with capillary columns. In 1958, Lovelock (68, 69) described a β-ray ionization detector which was extremely sensitive, and it has since become very popular for gas chromatography in the biochemical field. Argon is used as a carrier gas and is bombarded by α- or β-rays from a radioactive source inside the detector cell. This produces some argon ions and electrons which are accelerated by a high electric field of about 1000 v., reaching velocities sufficient to excite a large number of argon atoms to their metastable state. These excited species normally revert to ground state by emission of electromagnetic radiation. However, if they undergo collision with a molecule having an ionization potential below 11.6 e.v., the metastable argon gives up its excitation energy, thereby causing ionization of the colliding molecule. The ionization current produced in the electric field is amplified and recorded.

Another detector which is extensively used, both because of its high sensitivity, linearity of response, small volume, and simplicity of design is the hydrogen flame ionization detector developed by McWilliam and Dewar (70). The effluent from the column is mixed with hydrogen and fed through a jet where the gas is burned. Ions are formed in the flame by a mechanism of chemi-ionization (71). These are attracted to a collector electrode, thus producing an ionization current which is amplified and recorded.

Other detectors of interest include those based on electron capture (69, 72, 73), thermionic emission (74), and glow discharge (75).

The Column.—From Eq. 4 it is apparent that the retention volume is very much dependent on the amount of stationary liquid in the column. When columns with liquid loadings of the order of 0.5 to 3% are used, instead of the usual 15 to 30%, it becomes possible to decrease substantially retention volumes for compounds of low volatility. By taking advantage of the high sensitivity of the ionization detectors, the sample size can be reduced to such an extent that it will vaporize rapidly into the gas stream at temperatures which are 100 to 200° below the boiling points of the components. At the same time, improvements in instrument design and a greater variety of thermostable stationary phases have extended the range of application to high column temperatures.

An important development in gas chromatographic columns was initiated by Golay (76, 77) who proposed to use capillary tubing coated on the inner surface with the stationary phase instead of packed columns. These capillary columns, which are usually made from stainless steel, glass, or nylon tubing, range in length from 50 to several hundred feet. Efficiencies approaching a million theoretical plates have been achieved, but this is not directly comparable with the efficiencies of packed columns. The performance of coated capillary columns with respect to speed, column efficiency, and resolution has been thoroughly studied by Desty and co-workers (78–80). Purnell (55, 63) has shown that analyses performed with very long capillary columns of up to one-half million plates can be closely reproduced by a 7-ft. packed column of 5000 plates in about one-hundredth the time. Capillary columns have very low capacities and rarely operate efficiently if the quantity of any single component to be separated exceeds 10^{-7} Gm. A sample splitting device between the injection port and the column is, therefore, essential. There is still considerable controversy regarding the relative merits of packed columns and capillary columns. Undoubtedly, both types have their strengths and weaknesses. Although capillary columns have gained considerable popularity for separation of closely related compounds, especially in the petrochemical field, it is unlikely that they will replace the packed columns completely for analytical gas chromatography.

Tailing.—When polar materials are gas chromatographed on a nonpolar liquid phase, tailing can often cause considerable problems. It will distort the symmetry of the peaks, interfere with clean-cut separation, and make quantitative analysis very difficult. This is especially true if the concentration of liquid phase is low. The reason for this tailing effect is that most solid support materials are not completely inert, but contain active spots which cause adsorption. When adsorption takes place, the retention time is much influenced by the sample size. Large samples cause an earlier appearance of the front accompanied by a peak broadening. This has been attributed to a Langmuir adsorption isotherm which has reached the saturation limit (81). The effects of the solid support material on the retention volumes have been studied by Scholz and Brandt (82), Bens (83), Weinstein (84), and others. Adsorptive effects are due to weak van der Waals forces and strong hydrogen bonding forces, caused mainly by the presence of free hydroxyl groups on the support material. Several methods of deactivation have, therefore, aimed at removing the hydroxyl groups by reaction with dichlorodimethyl silane (85, 86), hexamethyldisilazane (81, 82), or by methylation (*cf.* 82).

Ormerod and Scott (87) have reported that silver coating of firebrick drastically reduces adsorptive effects. Others have added small amounts of fatty acids (4, 88), their salts (89), or various surface-active agents (81, 90, 91). These tend to deactivate hydrogen bonding sites, spread the stationary phase over the surface of the support, and possibly act as a "glue" to span the interface between the liquid and the solid (91). Addition of small amounts of a very polar stationary liquid to a nonpolar liquid will markedly reduce tailing without changing the retention values (81, 88, 92).

Temperature Programing.—Because of the band broadening factors expressed in the van Deemter equation, the peaks of a normal isothermal gas chromatogram become wider and lower as the retention time increases. Furthermore, it is possible for high boiling components to go undetected because of very long retention times (93–95). By increasing the temperature during the run of a chromatogram, it is possible to separate rapidly a mixture of components boiling over a wide range of temperature, and all solutes emerge as sharp, well-defined peaks of nearly the same shape (Fig. 2). With a linear temperature rise, each solute is eluted at a characteristic retention volume in the same way as for isothermal gas chromatography (96). The theoretical aspects of temperature-programed gas chromatography have been discussed by Giddings (97) and Said (98). Many commercial instruments have incorporated the feature of temperature programing. Dual column systems are sometimes used in order to maintain a stable base line by canceling the effects of increased column bleeding at higher temperatures (99).

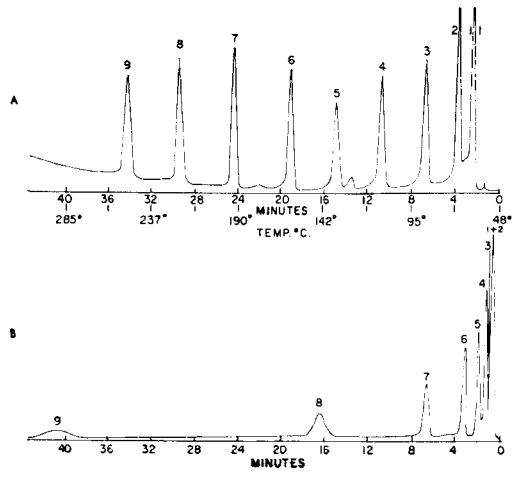


Fig. 2.—Gas chromatography of alcohols. A, temperature programing; B, isothermal gas chromatography at 165° of (*1*) methanol, (*2*) ethanol, (*3*) 1-propanol, (*4*) 1-butanol, (*5*) 1-pentanol, (*6*) cyclohexanol, (*7*) 1-octanol, (*8*) 1-decanol, and (*9*) 1-dodecanol. [After S. Dal Nogare and C. E. Bennett (93); courtesy of *Analytical Chemistry*.]

Sample Pyrolysis.—Organic substances, upon heating, undergo various decomposition reactions, the mechanism of which depends upon the structure of the molecules. Although the pyrolysis reactions are often extremely complex, they are reproducible under constant conditions. Pyrolysis has been used in conjunction with gas chromatography by several investigators (100–111) both for qualitative and quantitative analysis. The principle is simple. The product to be studied is applied to a filament which is placed in the stream of carrier gas. The filament is heated at a predetermined temperature for a few seconds by means of an electric current, and the pyrolysis products are carried by the gas stream to the column for separation. The method is particularly suited for analysis of polymers which, at a certain temperature, depolymerize to give the original monomers (111). However, numerous other compounds have been analyzed by this method, including amino acids, barbiturates, alkaloids, and steroids.

APPLICATIONS

Qualitative Analysis.—Identification of components in a mixture by gas chromatography is most frequently based on retention data which are related to the R_f value commonly used in paper chromatography. A comparison of the retention volumes (or times) of the unknown constituents with those of known compounds will often suffice to identify the former. However, the retention volumes are dependent on experimental conditions, many of which cannot be easily reproduced. It has, therefore, been recommended that retention data be reported in absolute units, such as specific retention volumes or partition coefficients. Somewhat simpler, from an experimental point of view, is the use of relative retention volumes (or times), where the effects of operating parameters are considerably reduced, depending mainly on the nature of the stationary phase and on the column temperature. The relative retention values are expressed by

$$r_{1,2} = \frac{V_{N1}}{V_{N2}} = \frac{V_{g1}}{V_{g2}} = \frac{V'_{R1}}{V'_{R2}} = \frac{t_1}{t_2} \quad \text{(Eq. 14)}$$

where the subscripts 1 and 2 stand for the sample component and the standard, respectively, V'_R is the net uncorrected retention volume, and t is the net retention time measured from the air peak to the sample peak.

Several modifications of this principle have been proposed. Evans and Smith (112), using a primary standard (*n*-nonane) and one or more internal standards, express the retention of the unknown relative to that of *n*-nonane as follows

$$R_{X9} = R_{XN} \cdot R_{N9} \qquad \text{(Eq. 15)}$$

where R_{X9} is the retention of the unknown relative to *n*-nonane, R_{XN} is its retention relative to the internal standard, and R_{N9} is the retention of the internal standard relative to *n*-nonane. Two internal standards are used whenever possible, one emerging on either side of the unknown, and the mean R_{X9} value is determined.

A closely related method is used by Kovats (113–116), who calculates a "retention index" on the basis of two reference standards.

The logarithm of the net retention volume of a substance is proportional to the free energy of solution of the solute molecules in the stationary phase. The free energy of solution is an approximately additive function of the groups constituting the molecule. Therefore, if the logarithm of the net retention volume (or relative retention volume) is plotted against some increasing property, such as the number of carbon atoms in a homologous series, a straight line is obtained. The slope of this line depends on the nature of the stationary phase (61, 117, 118).

In a complex mixture, there is always the possibility of two or more substances having the same retention characteristics on a particular column. This uncertainty with respect to separation and identification can be greatly reduced by making use of two or more columns of different polarity. Many methods are based on this principle. Lewis, Patton, and Kaye (119) used homologous series plots in which the logarithms of the elution times obtained on a nonpolar column were plotted against those obtained on a polar column. James (25) used log plots of the relative retention volumes for two stationary phases as a means of characterizing fatty acids. Brown (120) proposed a three-column method, basing the identification of the components of brown coal tar on the retention data measured with three different types of stationary liquids: a nonpolar liquid, an electron donor, and an electron acceptor. He defined an "affinity fraction," A, of the substance to be identified for each phase as

$$A_1 = r_1/(r_1 + r_2 + r_3)$$

where r_1, r_2, and r_3 are the retention volumes (or times) on the three stationary liquids under controlled conditions. The affinity fractions of a given substance in the three liquids were plotted on a triangular graph.

Because of the efforts required to achieve positive identification of complex mixtures solely on the basis of retention characteristics, many investigators have preferred to use gas chromatography in combination with chemical reactions or auxilliary instrumentation.

Walsh and Merritt (121) used a combination of retention volume and functional group analysis for the identification of chromatographic peaks. The functional group classification was accomplished by means of a stream splitting device attached to the exit tube from a thermal conductivity cell. As each chromatographic peak passed through the detector, the gas stream was split and the vapor collected in a set of reagents. A similar procedure was used by Dubois and Monkman (122). Various other chemical reactions, both before and after chromatographic separation, have been proposed for identification purposes (123, 124).

The combination of a gas chromatograph and a time-of-flight mass spectrometer is extremely useful for rapid and positive identification of complex mixtures. The mass spectra of the components can be scanned and recorded simultaneously with the running of the chromatograph (125, 126). The input system to the spectrometer can be designed so as to trap the sample from the column, letting it leak into the spectrometer long enough to allow the analog output system to record the spectrum.

Identification of gas chromatographic peaks by means of infrared or ultraviolet spectrophotometry is also extensively used (127–134). The fractions may be collected in a solvent or in solid potassium bromide and the appropriate analyses made, or a continuous scanning of the gaseous eluate may be carried out.

Another means of identification which is likely to become increasingly important is to use a combination of a selective and a nonselective detector. The electron capture detector is a selective detector having a high degree of specificity for halogenated compounds as well as for conjugated carbonyls and organometals (72, 135). Hydrocarbons, alcohols, amines, ketones, etc., produce virtually no response. Varadi (136) has developed an ionization detector which gives both qualitative and quantitative information. It is composed of a thermionic ionization detector unit for quantitative determination combined with a radio-frequency m/e analyzer, where the characteristic mass number of the gas component can be

determined. The signals from both detector parts are amplified and recorded on separate recorders.

Quantitative Analysis.—With a differential detector in combination with a potentiometric strip chart recorder, the substances appear as peaks having the dimensions of (millivolts) (time). The areas of these peaks are related to the concentration of component vapor in the carrier gas. The factor for converting peak area into weight varies with the detection system, the carrier gas, and the component to be determined. For accurate work, the detector response must be linear over a reasonable range of vapor concentration in the carrier gas. Accuracy and precision also depend on the sensitivity and stability of the detector and amplifier, on the method of sample application, and, in the case of capillary columns, on the linearity of the stream splitting system. Gas samples can be applied to the column with great accuracy by means of a gas sampling valve. For liquids and solids the problem is not nearly as simple, and many attempts have been made to develop reproducible sampling techniques. With the small samples required for very high boiling materials, especially in conjunction with ionization detectors and capillary columns, a simple syringe technique is commonly used although the sample size cannot be accurately reproduced. This usually requires an internal standard for calibration. A known amount of a reference substance is added to the sample and the mixture chromatographed. The composition of the sample is obtained from a standard curve based on chromatograms of several accurately known mixtures of the standard and the sample components. Two or more internal standards should be used if the sample contains substances with a wide range of boiling points (137).

Another method frequently used is referred to as internal normalization. If the detector response is approximately equal for all the components in a mixture, the total area under all the peaks is normalized to 100% and each peak calculated as a percentage of the total. If the detector response is markedly different for the different constituents in the sample, and this is usually the case, correction factors must be applied to each peak before they are added and proportioned.

Various methods have been proposed for the measurement of peak area. The simplest method which still gives reasonably good results when the peaks are symmetrical and well separated is triangulation, in which the area is assumed to be equal to the area of the triangle formed by the tangents through the points of inflection and the peak base. Greater precision can be achieved by means of a planimeter, although measurement errors for small peaks can be considerable. Besides, the method is slow and subject to operator fatigue. Based on the fact that gas chromatographic peaks, in the absence of adsorptive effects, closely approximate a Gaussian distribution curve, Bartlet and Smith (138) calculated peak areas from the peak height and the standard deviation for the curve by the formula

$$A = h\sigma\sqrt{2\pi} = 2.507\ h\sigma \qquad \text{(Eq. 16)}$$

where A is the area, h is the peak height, and σ the standard deviation for the normal distribution curve corresponding to the peak (= one-half peak width at 0.607 h).

The most accurate estimation of peak area may be obtained by a number of integrators based on analog or digital computation. A widely used type of analog computor is the ball and disk integrator which is relatively simple and inexpensive. It has an inherent accuracy within ±0.1% of full scale, and with proper care and operation a high degree of precision may be achieved (139). Recorder-integrator errors in gas chromatography area measurements have been studied by Orr (140). A formula has been developed to correct for these errors. With careful work, quantitative analysis by means of gas chromatography can give a high degree of precision and accuracy. This has been shown by many workers, but perhaps most clearly by Evans and Scott (141). Based on 110 consecutive determinations, the overall spread was 1%, the coefficient of variation 0.23%, and the 95% confidence limits ±0.35%. This compares very favorably with most instrumental methods of analysis.

Although gas chromatographic columns should be chosen so as to produce optimal separation, a complete resolution cannot always be obtained. Quantitative analysis may be made even if the peaks are overlapping. The problems involved and the methods used to overcome them have been discussed by several investigators (138, 142, 143).

ANALYSIS OF COMPOUNDS OF PHARMACEUTICAL INTEREST

It is only natural that the early applications of gas chromatography to pharmaceutical analysis have been concerned with very volatile

materials, such as anesthetic gases (144), alcohols, and other solvents, including water (145–149), nitroglycerin (145), essential oils, and their constituents (145, 150–155). In fact, gas chromatography has been a great impetus to the study of essential oils and perfumes, as well as the flavor and aroma substances present in food and beverages (156, 157). Because of the complex nature of these materials, no satisfactory method for a thorough, objective analysis was available until gas chromatography was developed. It is now possible, in a short time, to identify a volatile oil on the basis of the retention data and peak ratios of the chromatogram, and the components can be determined quantitatively. The method can also be used to detect adulterations and even to determine the geographical origin of the oil (158).

Gas chromatography has been used for monitoring the concentration of anesthetics in the patient's breath during the course of an operation (159) and to determine the absorption, distribution, and elimination of diethyl ether (160–162). Ether and chloroform, as well as various essential oil constituents, have been determined quantitatively in a variety of pharmaceutical preparations (150, 152, 163, 164).

Gas chromatography was developed in connection with the separation of organic acids, and a tremendous amount of work has been done in biochemical research involving studies of fatty acids and lipids and their metabolism in man and animals. The fatty acids are usually converted to the methyl esters by one of several methods (165) although the free acids may also be gas chromatographed (4, 166–168). Various polyesters are particularly selective for separation and identification of fatty acid esters (169, 170). The specific distribution of fatty acids in the glycerides of vegetable fats has been studied by means of gas chromatography (171) which promises to be an exceptionally useful method for quality control of fats and oils, including identification, detection of adulteration, and misbranding (172), and determination of the amounts of free fatty acids present (173). More recently it has been shown that the triglycerides may be gas chromatographed directly (174–176). Whether this method will prove to be more valuable than the standard procedure based on the fatty acid methyl esters still remains to be seen.

The resin components of marihuana (*Cannabis sativa*) have been separated by Kingston and Kirk (177) and by Farmilo and Davis (178). In addition to the major component, cannabinol, a large number of smaller peaks illustrate the complex nature of this material. The method appears to be of value for determination of the origin of seized marihuana.

Although aliphatic amines were among the first substances gas chromatographed by James (61), it is only recently that the method has been applied to pharmaceutically important amines. Brochmann-Hanssen and Svendsen (179, 180) have gas chromatographed a number of commonly used sympathomimetic amines on silicone rubber SE-30 and achieved excellent separation in most instances. The reaction between amines and ketones is useful for identification purposes and to separate certain isomers, such as ephedrine and pseudoephedrine. The catechol amines, epinephrine and norepinephrine, have been gas chromatographed after treatment of the triacetyl derivatives with hexamethyldisilazane.

Fales and Pisano (181) have also separated a number of biologically important amines including histamine, seratonine, tyramine, and some sympathomimetics.

In a recent publication Parker, Fontan, and Kirk (182) reported gas chromatographic separation and identification of a large number of tranquilizers, including phenothiazine derivatives, diphenylmethane derivatives, butanediols, propanediols, and others. No peaks were observed after application of rauwolfia alkaloids. Occasionally, following storage of some of the tranquilizers, new small secondary peaks appeared which increased in height with the passage of time. This suggested a possible application of the method to quality control during manufacture.

Gas chromatography of phenothiazine derivatives has also been reported by Anders and Mannering (183).

During the past year, the barbiturates and some related substances have been successfully gas chromatographed on various stationary liquids in low concentration (184–186). Not all compounds can be separated on the same column, but by using two columns, one with a nonpolar phase such as Apiezon L and one with a moderately polar polyester, a large number of possible combinations can easily be separated. The method has been used for identification of pharmaceutical preparations and for the study of the metabolism and excretion of barbiturates in humans (186, 187). The partial transformation of phenobarbital to *p*-hydroxyphenobarbital, and the demethylation of N-methyl substituted barbituric acid derivatives have been confirmed

by means of gas chromatography.

Stainier and Gloesener (188) have analyzed certain ester alkaloids and related synthetic drugs by hydrolysis and gas chromatography of the liberated alcohols. However, such pretreatment is usually not necessary. The volatile tobacco alkaloids can easily be gas chromatographed, as was shown by Quin (189–191) in 1958. More surprising is the fact that many alkaloids of high molecular weights and relatively low stability can be gas chromatographed as such. This was first demonstrated by Lloyd, *et al.* (192), who gas chromatographed 45 different alkaloids on low-loaded columns of silicone rubber SE-30. In this way, strychnine was separated from brucine, quinine from cinchonine, and the various major alkaloids in opium were separated from one another. Eddy, *et al.* (193), used this method for the determination of the geographical origin of opium. Brochmann-Hanssen and Svendsen (194) also applied gas chromatography to the separation and study of alkaloids in several crude drugs. Alkaloidal salts were gas chromatographed directly without prior isolation of the base. The salts dissociated in the flash heater which was maintained at about 325°, and the alkaloids were eluted as the free bases. The decompositions sometimes observed when certain alkaloids were subjected to gas chromatography were discussed (194).

Few classes of drugs are of greater importance than the steroids. Therefore, when it was shown in 1960 (195, 196) that steroids could be analyzed by gas chromatography with little or no decomposition, this created a tremendous interest among biochemists and pharmaceutical chemists. A new tool had become available for research in this area, and during the last two years an impressive amount of work has been done with it, particularly by Horning and his co-workers. Silicone rubber SE-30, the more polar and more selective fluorosilicone fluid QF 1-0065 (197), and various heat-stable polyesters (198, 199) have been found to be satisfactory stationary phases for gas chromatography of steroids. The method has been applied to corticosteroids (200, 201), androgens (199, 202), estrogens (202, 203), steroidal amines (204), sapogenins (205), cholesterol (86, 196), bile acids (86, 202), vitamin D (207), etc.

Synthetic estrogens, such as diethylstilbestrol, have been estimated in extracts of tissue, feces, and urine (208).

Several attempts have been made over the years to separate mixtures of amino acids by gas chromatography. The amino acids are not sufficiently volatile to be gas chromatographed directly, but may be converted to volatile products by various reactions. These include oxidation to aldehydes (209, 210), reaction of the amino group with acetic anhydride or trifluoroacetic anhydride followed by esterification of the carboxyl group (211–215), or treatment with hexamethyldisilazane (216). Pisano, *et al.* (217), have gas chromatographed the phenylthiohydantoin and the dinitrophenyl derivatives of amino acids.

The best results so far appear to have been obtained by Johnson, *et al.* (215, 218), who have separated a large number of amino acids as the *n*-amyl esters of the N-acetylamino acids. The method has been applied to hydrolysates of peptides and proteins (218).

Carbohydrates, including mono-, di-, and trisaccharides, as well as simple glycosides, have been gas chromatographed, either as the methyl ethers (219–223), the acetates (224–226), or the trimethylsilyl ethers (227). Using a column of fluorosilicone fluid QF 1-0065, Horning, *et al.* (226), separated the tetraacetates of α- and β-glucose. No separation was achieved with the less selective silicone rubber SE-30.

SPECIAL PROBLEMS IN PHARMACEUTICAL ANALYSIS

Determination of Purity.—An important aspect of pharmaceutical analysis is concerned with the identification and determination of trace impurities, and gas chromatography is excellently suited for this purpose. Bennett, *et al.* (228), have determined trace amounts of impurities in various solvents with a reproducibility of better than 2% for well-resolved peaks. Figure 3 illustrates the importance of instrument sensitivity. Chromatograms I and II are typical runs obtained with conventional equipment. Runs III and IV were made at a one hundred-fold increase in sensitivity. The total concentration of impurities in the cyclohexane sample was 0.3% and could not be detected by infrared analysis. Maruyama and Seno (229) determined trace amounts of methanol in ethanol and reported a detection limit of 0.005%. Impurities in chloroform have been studied by Gloesener (230). An interesting report by Iguchi, *et al.* (231), describes the application of gas chromatography to the determination of trace amounts of arsenic. The sample is reduced with zinc and acid, and the arsine is gas chromatographed with hydrogen as the carrier gas. As little as 1 mcg. could be detected, while the limit for quantitative determination was 4 mcg.

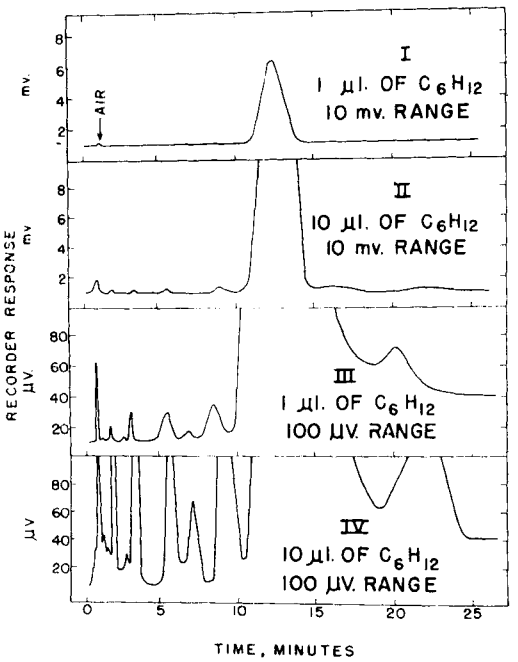


Fig. 3.—Chromatograms of cyclohexane at 10 mv. and 100-µv. full-scale sensitivity ranges. [After C. E. Bennett, *et al.* (228); courtesy of *Analytical Chemistry*.]

Using a column of toluene on granular Teflon and a temperature of −78°, Bergmann and Martin (232) separated bromide and chloride as the hydrogen halides. The sample was added to 70% sulfuric acid, whereby the halide ions were converted to the covalent hydrogen halides which were collected in a nitrogen trap and then gas chromatographed. Concentrations down to 0.01 p.p.m. in water could be detected. The method also permitted easy determination of trace amounts of chloride in bromide, a difficult analysis by other methods.

Much work has been done in the field of pesticide residues on fruits and vegetables. The electron capture detector is excellent for analysis of chlorinated pesticides because it permits determination of submicrogram amounts of the pesticides in the presence of a considerable excess of coextracted material (233). Coulson, *et al.* (234), used a coulometric detection system for quantitative analysis of halogenated pesticide residues.

A concentration technique is frequently needed in order to eliminate interference from other components in the sample (235, 236). Hornstein, *et al.* (173), determined the free fatty acids in fat emulsions by adsorption on an anionic exchange resin, esterification of the acids on the resin by means of methanol and hydrochloric acid, and gas chromatography of the isolated methyl esters. Trace components in an essential oil were determined after all polar matrix compounds had been removed by adsorption on silica gel (237).

Elemental Analysis.—Rapid methods have been developed for determination of carbon and hydrogen by means of gas-solid chromatography (238, 239). The organic sample is burned in a stream of oxygen, and the resulting carbon dioxide and water vapor are passed through a calcium carbide tube which converts the water to acetylene. The gases are concentrated in a freeze trap and then vaporized and swept into the chromatographic system by the carrier gas. The method is claimed to be very precise (0.5% for carbon and 0.1% for hydrogen) and more rapid than conventional methods. The time of analysis is about 20 minutes for a single determination and about 10 minutes each for a continuous series of analyses including area measurements (238). Vogel and Quattrone (240) have modified the method somewhat. Using gas-liquid chromatography with dodecyl phthalate as the stationary liquid and oxygen as the carrier gas, they eliminated the need for isolating the products of combustion from the combustion atmosphere.

Direct gas chromatographic analysis of oxygen in organic compounds has been reported by Goetz (241) and by Suchanec (242). Oxygen is converted to carbon monoxide at high temperatures by means of charcoal (241) or a platinum-carbon mixture (242). Suchanec reports a standard deviation of ±0.17%. Gas chromatographic methods are also available for determination of organically bound nitrogen (243) and sulfur (244).

Determination of Surface Area.—Surface area and particle size determinations are often of considerable importance in studies related to formulation of pharmaceutical dosage forms. This is a field in which gas chromatography has been particularly useful. In 1958 Nelsen and Eggertsen (245) described a method for determination of surface area based on adsorption chromatography.

The column packing is the sample whose surface area is to be determined and the mobile gas is a mixture of a suitable adsorbate (nitrogen) and an inert gas (helium). A known mixture of nitrogen and helium is passed through the sample in a U-shaped tube, the effluent being monitored by a thermal conductivity detection system. When a stable base line is obtained, the sample tube is immersed in liquid nitrogen. The sudden cooling causes absorption of nitrogen

and a corresponding negative peak on the recorder chart. After the equilibrium condition is established again, as indicated by the return of the recorder pen to the base line, the coolant is removed, causing desorption of nitrogen from the sample and a positive peak on the chart. Both peak areas are of the same size, being a function of the amount of nitrogen absorbed by the sample, and may be used for surface area measurements by either an absolute or a comparative method. Modifications of this method have been described by Roth and Ellwood (246) and by Stock (247).

Separation of Isomers.—A problem which has intrigued pharmaceutical chemists for a long time is the separation of isomers, particularly optical isomers.

Partial separation of racemic mixtures has been reported by means of an optically active stationary liquid such as *d*-tartaric acid ethyl ester (248). However, best results seem to have been achieved so far by converting the optical isomers to diastereomeric derivatives prior to the gas chromatographic analysis. In this way, Casanova and Corey (249) separated the optical isomers of camphor as ketals prepared from D(-)-2,3-butanediol. The choice of liquid stationary phase was very critical, as was also shown in the separation of the four stereoisomeric racemates of menthol. Using Hyprose SP-80 [octakis (2-hydroxypropyl) sucrose] and a 12-ft. column, Moore and Kossay (250) were able to determine quantitatively menthol, isomenthol, neomenthol, and neoisomenthol obtained by catalytic hydrogenation of thymol. The amounts of the various isomers in peppermint oil can also be determined (158, 251). Optical isomers are not separated by this procedure.

Bates, *et al.* (252), have isolated and characterized the four stereoisomeric farnesols on a 5-ft. column containing 20% of polyethylene glycol 20M. The isomers of ionone and methylionone have likewise been separated by gas chromatography (253, 254). One may expect considerable work in this field in the future, particularly in regard to high molecular weight drugs.

Process Control.—Chemical analyses for process control must be rapid and dependable, and the instrumentation must be rugged and preferably suitable for automatic operation. Gas chromatography satisfies most of these requirements, and during the last few years it has become an important tool for monitoring chemical reactions. The method is not continuous in the strict sense, since the separation does require a definite time. However, it can be made automatic in that it is carried out according to a predetermined schedule without manual operation. The instrument itself samples the stream, passes the sample through the column, and records the components as they emerge (255–260). High speed chromatography is especially important for this type of application. Several commercial instruments for process analysis are available.

Miscellaneous Applications.—Gas chromatography has been a valuable aid in the determination of structures of organic molecules, particularly in conjunction with pyrolysis or other degradation reactions. Formyl and acetyl groups in digitalis glycosides have been identified by gas chromatography after transesterification with methanol (261, 262). Lloyd, *et al.* (263), used the method to determine the position of aromatic methoxyl groups in alkaloids related to powelline, while Cox (264) identified the side chains of steroids by pyrolysis followed by gas chromatography. Others have been able to use gas chromatographic techniques for determination of the amino acid sequence in proteins (265) and the arrangement of fatty acids in triglycerides (171). Dhont (108) has made a systematic study of the correlation between structure and the pyrolysis products of organic compounds.

Because of its speed and selectivity, the gas chromatographic method is well suited for the study of reaction mechanisms and reaction kinetics, both with regard to the synthesis of drugs, their *in vitro* deterioration (266), and their biotransformation. The effects of experimental parameters on the yield of a synthetic procedure may be readily evaluated and optimum conditions established.

CONCLUSIONS

No attempt has been made to exhaust completely the existing literature with reference to the applications of gas chromatography to pharmaceutical and related analysis. However, it should be apparent that the method has become a powerful tool for research as well as routine analytical work. The use of gas chromatography for high molecular weight compounds of low volatility, a very recent adaptation, has greatly stimulated interest among pharmaceutical chemists, since the vast majority of drugs fall in this category. It is important to realize that many chemical substances which are quite unstable even at

room temperature in the presence of air and moisture, frequently hold up very well in the vapor state in the inert atmosphere of a gas chromatographic column. Where the substance to be analyzed is unstable at the elevated temperature required for volatilization, stable, volatile derivatives can often be prepared, or the pyrolysis technique may be applied.

The pharmaceutical industry is making use of gas chromatography on a rapidly increasing scale and it is not unreasonable to assume that the method may be recognized by the official compendia in the near future.

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Research Articles

Substituted Heterocyclic Thioureas I

Antitubercular Activity

By ARTHUR C. GLASSER and RICHARD M. DOUGHTY

A series of substituted 2-pyridyl and 4-(1-phenyl-2,3-dimethyl)-5-pyrazolone thioureas have been synthesized and tested for their antitubercular activity against *Mycobacterium tuberculosis* in Dubos medium. The minimum inhibitory concentrations of the compounds studied ranged from 10 mg. per 100 ml. to 0.16 mg. per 100 ml.

THIOUREA and its simple derivatives have been shown to have a limited tuberculostatic activity (1). *p*-Aminophenyl alkyl ethers also have a definite inhibitory effect (2), and when the two classes of compounds are combined through N-substituted thioureas this activity is enhanced (3–7). The tuberculostatic activity of the thioureas has been reviewed by Schroeder (8). This study is concerned with the influence on tuberculostatic activity by the substitution of the thiourea molecule with heterocyclic rings such as 2-pyridyl- or 4-(1-phenyl-2, 3-dimethyl)-5-pyrazolone in conjunction with alkyl or *p*-alkoxyphenyl substitution. The 1-*p*-alkoxy-

Received March 19, 1962, from the College of Pharmacy, University of Kentucky, Lexington.
Accepted for publication April 23, 1962.
This investigation was supported in part by research grant E-4089, National Institutes of Health, Public Health Service.

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Antitubercular Activity

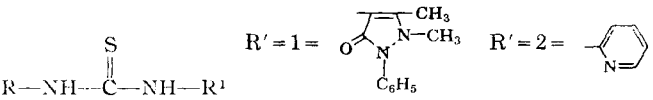
By ARTHUR C. GLASSER and RICHARD M. DOUGHTY

A series of substituted 2-pyridyl and 4-(1-phenyl-2,3-dimethyl)-5-pyrazolone thioureas have been synthesized and tested for their antitubercular activity against *Mycobacterium tuberculosis* in Dubos medium. The minimum inhibitory concentrations of the compounds studied ranged from 10 mg. per 100 ml. to 0.16 mg. per 100 ml.

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Accepted for publication April 23, 1962.
This investigation was supported in part by research grant E-4089, National Institutes of Health, Public Health Service.

TABLE I.—SUBSTITUTED HETEROCYCLIC THIOUREAS



No.	R	R′	M.p.[a], °C.	R.S.[b]	Yield[c], %	Formula	N Analyses[d] Calcd.	N Analyses[d] Found	Min. Inhib. Concn., mg./100 ml.
1	C_6H_5	1	207–08[e]	E	90	$C_{18}H_{18}N_4OS$	16.5	16.3	2.5
2	C_6H_{11}	1	252–53d decompn.	C	82	$C_{18}H_{24}N_4OS$	16.3	16.8	5
3	4-$CH_3OC_6H_4$	1	200–01[f]	M	89	$C_{19}H_{20}N_4O_2S$	15.2	15.3	2.5
4	4-$C_2H_5OC_6H_4$	1	200–01.5[g]	M	87	$C_{20}H_{22}N_4O_2S$	14.7	14.7	2.5
5	4-*n*-$C_3H_7OC_6H_4$	1	198–200d	AM	69	$C_{21}H_{24}N_4O_2S$	14.1	14.6	2.5
6	4-*i*-$C_3H_7OC_6H_4$	1	202–03d	M	89	$C_{21}H_{24}N_4O_2S$	14.1	14.3	0.16
7	4-*n*-$C_4H_9OC_6H_4$	1	201–02.5	T	87	$C_{22}H_{26}N_4O_2S$	13.6	13.2	10
8	4-*sec*-$C_4H_9OC_6H_4$	1	195–96d	MI	70	$C_{22}H_{26}N_4O_2S$	13.6	13.6	5
9	4-*n*-$C_5H_{11}OC_6H_4$	1	173–75d	C	81	$C_{23}H_{28}N_4O_2S$	13.2	12.7	5
10	4-*sec*-$C_5H_{11}OC_6H_4$	1	180–81d	C	71	$C_{23}H_{28}N_4O_2S$	13.2	13.0	2.5
11	C_2H_5	2	122.5–23	A	65	$C_8H_{11}N_3S$	23.2	22.9	2.5
12	*n*-C_3H_7	2	87.5–88.5	A	68	$C_9H_{13}N_3S$	21.5	21.3	5
13	*i*-C_3H_7	2	129.5–30[h]	A	78	$C_9H_{13}N_3S$	21.5	21.0	2.5
14	C_3H_5	2	101–02[i]	A	90	$C_9H_{11}N_3S$	21.7	21.2	5
15	*n*-C_4H_9	2	83–84	A	84	$C_{10}H_{15}N_3S$	20.5	20.0	10
16	*n*-C_5H_{17}	2	80–82	A	75	$C_{14}H_{23}N_3S$	15.8	15.6	1.25
17	C_6H_5	2	177–78[j]	B	92	$C_{12}H_{11}N_3S$	18.3	18.1	1.25
18	C_6H_{11}	2	147–48	A	63	$C_{12}H_{17}N_3S$	17.8	17.5	2.5

[a] Fisher-Johns Block, uncorrected. [b] Recrystallization solvents: A-alcohol, B-acetone, C-Methanol, E-Ether, I-Isooctane, M-Methylcellosolve, T-Tolune. [c] Yields based on recrystallized product. [d] Weiler and Strauss, Oxford, England. [e] Reported, Takahasi, T., *Yakugaku Zasshi* **79,** 162(1959), 208°; through *Chem. Abstr.*, **53,** 13128e(1959). [f] Reported, *ibid.*, 199°. [g] Reported, *ibid.*, 198°. [h] Reported, Roy, A., and Guha, R. C., *J. Sc. Research (India)*, **9B,** 9262(1950), 129–130°. [i] Reported, *ibid*, 100–101°. [j] Reported, *ibid.*, 167°.

phenyl-3-(2-pyridyl) thioureas have been investigated (5). With our observation of the activity of the 1-*n*-octyl-3-(2-pyridyl) thiourea it was decided to investigate the other members of the 2-pyridyl series along with the 5-pyrazolone series.

The *in vitro* determination of the tuberculostatic activity was carried out using the virulent H37Rv strain of *M. tuberculosis* grown on Dubos medium with added Tween 80 and beef serum. A comparison of the biological activities as seen from Table I shows the inhibitory concentrations range in activity from a low value of 10 mg./100 ml. to an upper limit of 0.16 mg./100 ml. Maximum activity of the 2-pyridyl series rests in the phenyl and *n*-octyl derivatives, while maximum for the 5-pyrazolone series is shown by the isopropoxyphenyl derivative. In the *p*-alkoxyphenyl derivatives the increased activity of the lower alkyl chains is in keeping with observations made on the 4,4′-dialkoxythiocarbanilides (9).

Synthesis of the thioureas involved the reaction of 2-aminopyridine or 4-aminoantipyrine with the appropriate isothiocyanate in absolute alcohol or thiophene free benzene (10). The alkyl isothiocyanates were prepared by the general method of Moore (11). The aryl isothiocyanates were made with some modification following the process of Van der Kirk (12), or the action of acetic anhydride on the appropriate thiocarbanilide (13). The *p*-alkoxyaniline derivatives needed for the isothiocyanate syntheses were prepared by the method of Bucchi (14).

EXPERIMENTAL

Biological Test Method.—The culture used was the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* obtained from the American Type Culture Collection.[1] The organism to be used as inoculant was propagated in Dubos broth base.[2] These cultures were grown in 100 ml. of broth in aluminum foil capped Erlenmeyer flasks for 2 weeks before inoculating tubes in the dilution series. Incubation was at 37° for all cultures and dilution tubes.

In the dilution series 4.5 ml. of Dubos broth base, enriched with 10% Dubos Medium Serum,[2] was placed in all tubes except the first tube of the series which received initially 9.0 ml. of the enriched medium. Ten milligrams of the compounds per milliliter of a solvent consisting of equal parts of propylene glycol, Tween 80, ethanol and distilled water was diluted to 10 ml. with nonenriched Dubos broth base. Using a sterile Swinny hypodermic adaptor and a membrane filter, Millipore HA type,[3] 1.0 ml. was aseptically filtered into the first tube of a series. These were mixed by drawing up portions of the medium into a sterile syringe, then 4.5 ml. was withdrawn aseptically from the first tube and placed in the second. This was mixed as before and 4.5 ml. transferred to the next tube. This was continued through all tubes of a specific series; 4.5 ml. was discarded from the last tube in the series. This resulted in increasing dilutions of 2× magnitude

[1] American Type Culture Collection, 2112 M Street N. W., Washington 7, D. C.
[2] Difco Laboratories, Detroit 2, Mich.
[3] Millipore Filter Corp., Bedford, Mass.

from 10 mg. % in the first tube to 0.0195 mg. % in the last tube of a ten tube series.

One-half milliliter of the propagation culture was added to each tube of the diluted agent in Dubos medium in a series. Centrifugation in a Hopkins vaccine tube of the propagation cultures showed the concentration of organisms to average about 0.0007 ml. packed cells per 0.5 ml. of culture.

All tubes in the tests were read at the end of 10 days and again at the end of 2 weeks. There was little difference in the two readings. First, tubes which developed a characteristic pellicle were read as positive for growth and noted. Acid-fast slides were prepared from doubtful positive tubes and also from the most dilute tubes showing macroscopically no growth. These were read "positive" if there were acid-fast bacilli in all fields, and "doubtful" if only a few acid-fast organisms found on the whole smear. Frequently, the microscopic examination resulted in lowering the evaluation of an agent. The compound 4,4'-diaminodiphenylsulfone was used as a standard for the activity of the compounds.

p-Alkoxyaniline Intermediates.—*p*-Phenetidine and *p*-anisidine are commercial products. Following the directions given by Bucchi (14), with the exception of an increased hydrolysis period to 8 hours, the following *p*-alkoxyanilines were prepared: *p*-propoxy (15), *p*-isopropoxy (14), *p*-*n*-butoxy (15), *p*-*n*-amoxy (15). *p*-*sec*-Butoxyaniline was prepared by this method in 80% yield, b.p. 150–151° (23 mm.).

Anal.—Calcd. for $C_{10}H_{15}NO$: N 8.47. Found: N 8.40.

p-*sec*-Amoxyaniline was also prepared by this method in 71% yield, b.p. 161–163° (23 mm.).

Anal.—Calcd. for $C_{11}H_{17}NO$: N 7.86. Found: N 7.79.

Aryl Isothiocyanates.—Phenyl isothiocyanate was a commercial product. The following *p*-alkoxyphenyl isothiocyanates were prepared by the action of sodium chloroacetate on the corresponding dithiocarbamates followed by decomposition of the resulting salt with zinc chloride (12): *p*-methoxyphenyl (12), *p*-ethoxyphenyl (12), *p*-propoxyphenyl (5), *p*-isopropoxyphenyl (5), *p*-butoxyphenyl (5), and *p*-amoxyphenyl (5). Prepared in this same manner was *p*-*sec*-butoxyphenyl isothiocyanate, b.p. 176–178° (28 mm.).

Anal.—Calcd. for $C_{11}H_{13}NOS$: N 6.77. Found: N 6.91.

Also prepared in this fashion was *p*-*sec*-amoxyphenyl isothiocyanate b.p. 157–158° (3mm.).

Anal.—Calcd. for $C_{12}H_{15}NOS$: N 6.36. Found: N 6.27.

2-Pyridyl Thioureas.—A 10-Gm. (0.07 mole) quantity of cyclohexyl isothiocyanate (16) was refluxed with 6.7 Gm. (0.07 mole) of 2-aminopyridine in 15 ml. of thiophene free benzene for 1 hour. The reaction mixture was concentrated through removal of 10 ml. of benzene and allowed to stand overnight. The resulting solid mass was recrystallized from alcohol to yield 10.5 Gm. (63%) of 1-cyclohexyl-3-(2-pyridyl) thiourea, m.p. 147–148°.

Anal.—Calcd. for $C_{12}H_{17}N_3S$: N 17.8. Found: N 17.4.

4-(1-Phenyl-2,3-dimethyl)-5-pyrazolone Thioureas.—*p*-Isopropoxyphenyl isothiocyanate weighing 1.93 Gm. (0.01 mole) and 2.03 Gm. (0.01 mole) of 4-aminoantipyrine were refluxed for ½ hour in 50 ml. of absolute alcohol. The reaction mixture was chilled in ice and filtered to yield after drying in a vacuum desiccator 3.6 Gm. of the thiourea. Concentration of the mother liquor and chilling yielded another 0.3 Gm. for a total of 3.9 Gm. of crude product, m.p. 198–201°. The product recrystallized from methylcellosolve yielded 3.4 Gm. (89%) 1-*p*-isopropoxyphenyl-3-[4-(1-phenyl-2,3-dimethyl)-5-pyrazolone] thiourea, m.p. 202–203° decompn.

Anal.—Calcd. for $C_{21}H_{24}N_4O_2S$: N 14.1. Found: N 14.3.

The pyrazolone thioureas proved to be photosensitive, and upon exposure to light for various periods of time developed a yellow color.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product IV

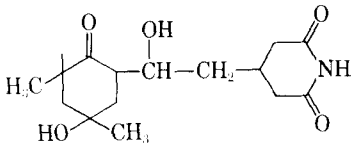
Screening Bulk Drug Stability and Defining the Assay of Choice

By EDWARD R. GARRETT† and ROSS R. HERR

Statistical evaluation of biological assays of bulk streptovitacin A subjected to 70° and room temperature for 60 days permitted the conclusion that the drug was stable in the bulk form. The assay of choice was defined against *Saccharomyces pastorianus* rather than against *Trichomonas vaginalis*. The point is made that operational potency is best defined by assay replication on various days but comparison of lot potencies and production procedures is best made by assay on one given day against the same standards.

WHEN a potential product results from pharmaceutical research, a facile and fast screen of stability in the bulk form of the drug is needed. When biological assays are the major method of potency determination, the stability study must be designed so that confidence in stability estimates must take into account the large inherent error in such assays. Such a design can also contribute supplementary information which can permit a choice of assay and evaluate the effects of variation among days or sampling procedures (1, 2, 3).

A case in point was the antitumor agent streptovitacin A (4–10)



The screening of stability was conducted on samples maintained at both 70° and room temperature. Only if significant differential degradation occurred would a more elegant accelerated stability study at several temperatures for purposes of stability prediction be warranted.

EXPERIMENTAL

Samples of streptovitacin A were weighed and placed in separate vials. One set of vials was subjected to 70° and the other was maintained at room temperature. At intervals, vials were removed and submitted for assay. The two assay procedures used are given in the literature (5), paper chromatographic separation on bioautograph development against *Saccharomyces pastorianus* and *Trichomonas vaginalis*.

RESULTS AND DISCUSSION

The analyses of variance (11) of the assays of streptovitacin A subjected to 70° and room temperature for 60 days are given in Tables I and II, the former for the *S. pastorianus* assay (5) and the latter for the *T. vaginalis* assay (5).

No significant differences between the assays of streptovitacin A maintained at room temperature and 70° for 60 days could be determined. These facts are graphically shown in Fig. 1. No significant trend of assays with days of sampling was apparent for the material maintained at 70°. The differ-

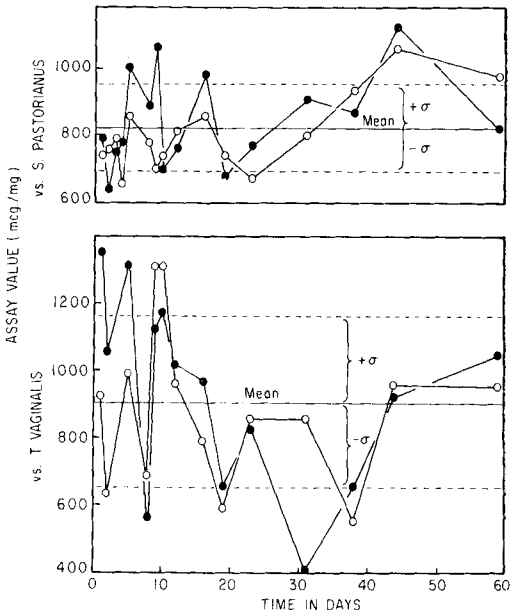


Fig. 1.—The assay of solid streptovitacin A at 70°, ●, and at room temperature, ⊙, as a function of time. The solid line represents the mean of the assays; the dashed lines represent the limits of the mean ± standard deviation.

Received September 5, 1961, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication October 18, 1961.
The technical assistance of Mr. E. Markovich in the operation of the E102 Burroughs Digital Computer is gratefully acknowledged. The authors are indebted to Dr. W. T. Sokolski and associates for the microbiological assays.
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TABLE I.—ANALYSIS OF VARIANCE OF STREPTOVITACIN A MAINTAINED AT TWO DIFFERENT TEMPERATURES[a] AND ASSAYED ON VARIOUS DAYS (ASSAY AGAINST *S. Pastorianus*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days[b]	323,242	13	24,864[c]	$2\sigma_D^2 + \sigma_E^2$
Temperatures	11,401	1	11,401[d]	$14\sigma_T^2 + \sigma_E^2$
Error	117,013	13	9,001	σ_E^2
Total[e]	451,657	27	16,728	

[a] Average of room temperature samples: 813; average of 70° samples: 854; overall average, $\bar{x} = 834$. [b] Estimated variance, $\sigma_D^2 = 7{,}930$. [c] F test: days:error = 2.77; 5% F = 2.6 ∴ sign. @ 5% level. [d] F test: temp.:error = 1.27; 5% F = 4.7 ∴ no significance. [e] Estimate of variance among days and error: $\sigma_E^2 + \sigma_D^2 = 16{,}931$

$$\therefore \sigma = 130 \therefore \frac{\sigma}{\bar{x}} = \frac{130}{834} \therefore 15.6\%$$

ences in the mean values of the assays of the 70° and room temperature materials can be attributed solely to assay variation.

Additional information may be obtained from the analyses of variance. For both assay methods, either against *S. pastorianus* or *T. vaginalis*, the assay variation among days is significant. In fact, when an assay is replicated on several days, the variation is twice that for replication on a given day.

The fact that this variation occurs in the same direction for samples run on the same day is apparent from inspection of the plots in the attached Fig. 1. When the 70° assays give results above the mean on given days, so do the room temperature assays; when they give results below the mean, so do the room temperature assays. That this synchronization of daily deviations from the mean for both samples is not a phenomenon attributable to some inherent variation in the day of sampling may be concluded from the analysis of variance for the total data from both assays as given in Table III.

The interaction days × assay is significant on the 1% level whereas variation among days and assays is not significant with respect to this interaction. Yet, as from Tables I and II, variation among days is significant within each assay. Thus it may be concluded that the variation among days is arbitrary with a particular assay; that there is not a common reason for the highs and lows of assay values *vs.* days to be synchronized for the several assays. Assays yield high and low values independently; results with a particular assay show a daily bias peculiar to that assay.

This is graphically shown in Fig. 1 where the assay values against *T. vaginalis* for a given day may be in excess of the mean, whereas those for *S. pastorianus* are just as likely to be less than their mean on that day. Thus no bias is apparent when the data from both assays are averaged.

These facts permit one point to be stressed. Variation among days of assay is a phenomenon common to microbiological methods (12, 13). Fundamental investigations of the causes of diurnal microorganism or biological variation may eliminate the source of increased error among assays replicated on various days. In this particular study, such elimination would have halved the assay variance.

If an assay is to serve as an operational definition of drug potency and since potency may be evaluated on any arbitrary day, replicate assays on several days may most adequately define the drug. How-

TABLE II.—ANALYSIS OF VARIANCE OF STREPTOVITACIN A MAINTAINED AT TWO DIFFERENT TEMPERATURES[a] AND ASSAYED ON VARIOUS DAYS (ASSAY AGAINST *T. Vaginalis*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days[b]	1,359,035	13	104,541[c]	$\sigma_E^2 + 2\sigma_D^2$
Temperature	19,189	1	19,189[d]	$\sigma_E^2 + 14\sigma_T^2$
Error	384,431	13	29,572	σ_E^2
Total[e]	1,762,654	22	65,283	

[a] Average of room temperature samples: 880; average of 70° samples: 924; overall average: 906. [b] Estimated variance; $\sigma_D^2 = 34{,}485$. [c] F test; days:error = 3.54; 5% F = 2.6 ∴ sign @ 5% level. [d] F test; temp. error <1 ∴ no significance. [e] Estimate of variance among days and error: $\sigma_E^2 + \sigma_D^2 = 64{,}057$.

$$\therefore \sigma = 253 \therefore \frac{\sigma}{\bar{x}} = \frac{253}{906} \therefore 27.9\%.$$

TABLE III.—ANALYSIS OF VARIANCE OF TWO ASSAYS BY DAYS OF ASSAY[a] WITH 70° AND ROOM TEMPERATURE MATERIAL CONSIDERED AS REPLICATES (ASSAYS AGAINST *S. Pastorianus* AND *T. Vaginalis*)

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Quantities Estimated by Mean Squares
Days	873,545	13	67,196[b]	$\sigma_E^2 + 4\sigma_D^2 + 2\sigma_{D\times A}^2$
Assays	73,732	1	73,732[b]	$\sigma_E^2 + 28\sigma_D^2 + 2\sigma_{D\times A}^2$
Day × Assay	808,732	13	62,210[c]	$\sigma_E^2 + 2\sigma_{D\times A}^2$
Error	532,034	28	19,001	σ_E^2
Total	2,288,000	55	41,601	

[a] Average among days: 883; average among assays: 906; over-all average: 870. [b] Days and assays are not significant with respect to D × A interaction. [c] F test: D × A:error = 3.22; 1% F = 2.9 ∴ sign. on 1% level; estimated variance $\sigma_{D\times A}^2 = 21{,}600$.

ever, drug lots or development procedures to give higher purity or yield would be most accurately compared by assay on one day with the same standard curves. Of course, the possibility of sample × assay × day interaction must not be ignored.

CONCLUSIONS

A screening procedure was used to evaluate the stability of a drug in the bulk form, and assays of streptovitacin A at 70° and room temperature for 60 days were compared. It is concluded that streptovitacin A is stable in the bulk form and is not subject to any thermal degradation which results in loss of biological potency.

The quantitative papergram assay against *S. pastorianus* for streptovitacin A is the method of choice rather than against *T. vaginalis*. The standard deviation, per cent of the mean, for the estimation of error of a single assay is 16% for the former, and 28% for the latter.

The variation of an assay within a single day is only one-half of the variation of that assay on various days. This phenomenon of daily variation has to do with the bias of the assay and not the day of sampling. Elimination of such daily assay variation could halve the variance.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product V

Importance of Assay Validity, a Case in Point

By EDWARD R. GARRETT†

Microbiological assays are used to measure drug potency but unwanted degradation products or impurities may positively interfere. A case in point is the tetracycline salts with normal moisture content in the cylinder-plate assay against *Bacillus subtilis*. Thermal degradation doubles the potency of tetracycline in this microbiological assay. Ultraviolet and infrared spectrophotometry indicate that aromatization and decarboxamidation are implicated. The resultant products have greater diffusivity and sufficient potency in the agar of the cylinder-plate assay method so that an *apparently* greater drug assay results. Dry tetracycline salts are thermally stable. Normal moisture content does not induce instability at usual storage temperatures. The kinetics of acid degradation of tetracycline phosphate and hydrochloride are the same.

The prediction of stability of drugs in pharmaceutical preparations (1) can be made only within the validity and reliability of the assay for drug. Previous papers in this series (2) have essentially considered the reliability or reproducibility of the assay.

The validity of an assay may be defined as its ability to measure that which it is supposed to measure. When biological assays are necessary, it is frequently assumed that degradation products of the drug do not demonstrate potency under the conditions of assay. This is not necessarily so. Biological assays are a complex sequence of physical, biological, and chemical effects. It is possible that the degradation products of a drug may not have the same pharmacological properties as the drug itself and yet give anomalous estimates of potency.

An interesting case in point is tetracycline and the assay in question is a classical microbiological method of antibiotic evaluation, the cylinder-plate assay (3) with *Bacillus subtilis* as the test organism.

EXPERIMENTAL

Biological Assay of Heat-Treated Tetracycline by Cylinder-Plate Assay.—Bulk tetracycline phosphate[1] with 2% moisture content was subjected to

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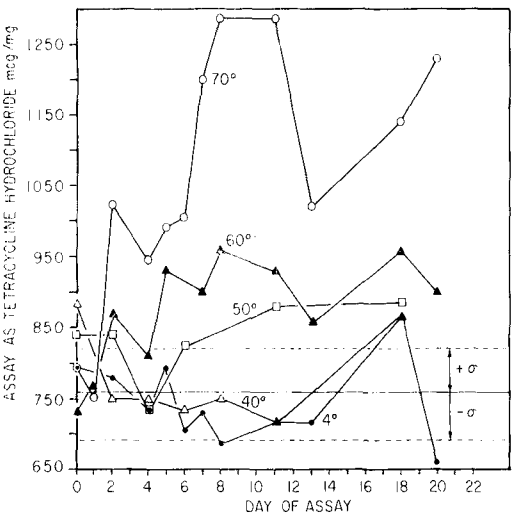


Fig. 1.—Cylinder-plate assay *vs. B. subtilis* of bulk tetracycline phosphate subjected to various temperatures. The solid line represents the mean of the 4° and 40° assays; the dashed lines represent the limits of the mean ± standard deviation.

70°, 60°, 50°, 40°, and 4° and assayed at intervals. The primary assay procedure was by the cylinder-plate method against *Bacillus subtilis* using tetracycline hydrochloride[2] as the standard. The collected assay data for the various temperatures are plotted as tetracycline hydrochloride against days of assay in Fig. 1.

Biological Assay of Heat-Treated Tetracycline Phosphate by Paper Chromatography.—Since the tetracycline phosphate was heat transformed to a material that had increased potency against *B. subtilis* in the cylinder-plate assay, it was deemed advisable to assay the material by paper chromatography against the same organism after the month of thermal treatment. The spot that moved like tetracycline was assayed as tetracycline against the standard. Table I gives the results.

TABLE I.—ASSAY AGAINST *B. Subtilis* OF PAPER CHROMATOGRAM OF TETRACYCLINE PHOSPHATE HEAT TREATED FOR ONE MONTH

Temp. of Treatment	mcg./mg. Assay A	mcg./mg. Assay B
70°	475	721
60°	767	900
50	958	925
40	829	1050
4°	925[a]	1050[a]

[a] This was the same material used as a standard in this assay.

Spectrophotometric Assay of Heat-Treated Tetracycline Phosphate.—The bulk tetracycline phosphate subjected to elevated temperatures was spectrophotometrically analyzed at pH values of 2.32, 4.88, and 9.25. Heat at 70° affected the spectra in that the apparent absorptivity at 218 mμ increased to a maximum of 45%, at 270 mμ to a maximum of 63%, and at 356 mμ decreased to 49.5% at pH 2.32.

[2] Marketed by The Upjohn Co. as Panmycin.

The absorbances at these various wavelengths and pH values became asymptotic with time at 70°. If the symbol for the asymptotic value at 70° is A_∞, then the first-order rate constants may be determined from the slope of plots based on the expression

$$\text{Log}\,(A_\infty - A) = -\text{kt}/2.303 + \text{constant}$$

Such plots for a given temperature were parallel, no matter the wavelength or the pH. Typical plots for 70°, 60°, 50°, and 40° treated material are given in Fig. 2. These were obtained at 218 mμ and at pH 2.32 in 0.005 *M* hydrochloric acid. The slopes of such plots are given in Table II.

TABLE II.—SLOPES OF POSSIBLE FIRST-ORDER HEAT DEGRADATION OF TETRACYCLINE PHOSPHATE

Temp.	Slope, $k/2.303$ in day^{-1}
70°	0.67
60°	0.0062
50°	0.0025[a]
40°	indeterminate

[a] Estimated but could be insignificant with respect to variability of the measurement.

Identification of Gaseous Product of Heat Degradation of Bulk Tetracycline Salts.—The bulk tetracycline phosphate darkened when subjected to temperature. The degree of darkening increased with temperature. The 70° material after a month's time was virtually dark brown. Within the bottles a gas pressure was generated. This gas was isolated and shown by infrared spectroscopy to be carbon dioxide.

Dried and normally hydrated tetracycline hydrochloride and phosphate were subjected to 70° temperature for several weeks. In *both* cases of hydration, decomposition to a gray-black mass was visually noted and CO_2 was determined to be present in both atmospheres. In both cases of the dried tetracycline, no discoloration was observed. It is thus concluded that CO_2 liberation and decomposition occur with moist tetracycline salt.

Solution Degradation of Tetracyclines.—Buffers were prepared of hydrochloric acid-potassium chloride for pH values of 1.2 and 2.2. The former was prepared with 250 ml. 0.2 *M* potassium chloride and

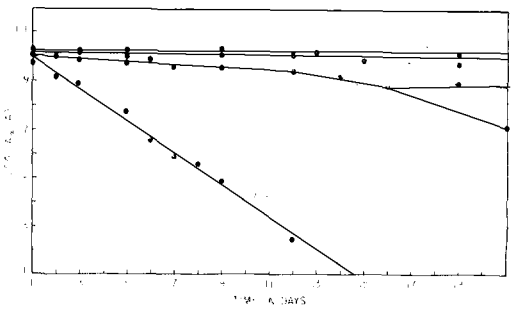


Fig. 2.—Apparent first-order rate plots for the thermal degradation of bulk tetracycline phosphate at various temperatures as based on ultraviolet spectrophotometry in pH 2 acid solution. The A_∞ is taken as the asymptotic absorbance with time of the 70° degraded material.

327.5 ml. 0.2 *M* hydrochloric acid and the latter with 250 ml. 0.2 *M* potassium chloride and 33.5 ml. 0.2 *M* hydrochloric acid (4). Both were brought up to volume in a 1-L. flask.

A 9.797 mg. quantity of tetracycline free base and 13.132 mg. tetracycline phosphate were each dissolved in 10 ml. dimethylformamide. Five milliliters of each solution were brought up to volume with 1.2 and 2.2 pH buffers, respectively. The resultant solutions were maintained at 30.3° and read at intervals on the Beckman model DU spectrophotometer at the maxima of tetracycline, 270 mμ and 356 mμ.

CALCULATIONS AND RESULTS

Anomalous Increase in Potency by Cylinder-Plate Assay of Heat-Degraded Tetracycline Salts.—The screening of bulk drug for instability can be readily effected by assaying heat-treated material at intervals of time. The assays of bulk tetracycline phosphate subjected to various temperatures are plotted as functions of time in Fig. 1.

Since the variation among assay values of the drug subjected to high temperatures was significantly outside the expected assay error, the mean and standard deviation (σ) in this figure is calculated solely from the 4° and 40° data.

The mean and standard deviation of the assay of this material as tetracycline were pooled from the entire month of study and are 760 and ±70 mcg./mg., respectively.

It is readily apparent that the material subjected to 70° and 60° significantly increased in apparent assay. The 70° material developed a potency of 1.7 times the mean value in 1.5 week's time. Although the increase in potency of the 50° material is not as statistically significant, it can be estimated as 1.15 the mean value. The 60° material developed a potency of 1.25 times the mean value.

There can be little doubt that the bulk tetracycline salt was transformed by heat to a new material that demonstrated increased potency *by this particular assay method.* These data do not permit any valid estimate of stability *as tetracycline,* since the assay method responds to degradation products other than tetracycline.

The ultraviolet spectrophotometric assay of the heat-degraded materials could provide a more quantitative estimate of rates of this transformation. Such estimates were given in Table II. Thus, if the ratio of rate constants at 10° intervals is chosen as $k_{70°}/k_{60°} \sim 100$ from Table II, the rate constant at 30° would be 6×10^{-9} day^{-1}. This would result in negligible spectrophotometric change in over a year at 30°. Even if the more disadvantageous ratio $k_{60°}/k_{50°} = 2.5$ were used, there would be negligible spectrophotometric change at 30° within a year.

The results of Table I confirm the spectrophotometric observations. The transformation of bulk tetracycline has a high thermal activation. Although the transformation has a high rate at 70°, it is much less at 60° and negligible at 50° and 40°. The best estimate from these data on the assumption that tetracycline is being assayed by this technique is that it is inherently stable in the bulk form at 30°.

Solution Degradation of Tetracycline Salts.—Plots of absorbance at 270 and 356 mμ, the maxima of tetracycline in acid media, against time in hours for tetracycline phosphate and hydrochloride at pH 2.2 and 1.2 are given in Fig. 3. The coincidence of data for phosphate and hydrochloride proves that both salt types undergo the same degradation at the same rate.

An example of the pseudo first-order plots of the absorbance data is given in Fig. 4 as based on the premise that

$$dx/dt = -k_H{}^+[H^+]x$$

where x is the concentration of tetracycline and $[H^+]$ is the hydrogen ion concentration. This integrates to

$$\log x/x_0 = -k_H{}^+[H^+]t/2.303 = -kt$$

where x_0 is the initial tetracycline concentration.

When absorbance is the measure of concentration, then

$$\log/A_t - A_\infty/ = -kt + \text{constant}$$

where A_t is the absorbance at time t and A_∞ is the asymptotic absorbance at infinite time and

$$k = k_H{}^+[H^+]/2.303$$

The rate of appearance of the chromophore at 270 mμ at pH 1.2 is 0.00211 and 0.00202 for hydrochloride and phosphate, respectively. The rate of disappearance of the chromophore at 356 mμ at pH 1.2 is 0.00200 for both.

The mean value of k in the fifth equation at pH 1.2 is thus 0.00203 and $k_H{}^+$ $[H^+] = 4.675\ 10^{-3}$ $hr.^{-1}$ Thus, $k_H{}^+ = 4.675.10^{-3}/10^{-1.2} = 7.41.10^{-2}$ 1/M/hr.

Thus, in general, the fraction of tetracycline remaining after acid degradation at a given pH and 30° may be calculated from the expression if specific acid catalysis is assumed

$$\log x/x_0 = -7.41.10^{-2}.10^{-pH}t/2.303 = 3.21.10^{-2}\,[H^+]t$$

It follows that a solution of tetracycline salt in 0.01 *M* hydrochloric acid undergoes 10% degrada-

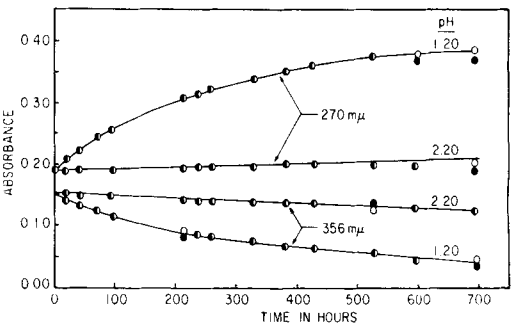


Fig. 3.—Change of absorbance with time for tetracycline hydrochloride, ⊙, and tetracycline phosphate, ●, at several acid pH values at 30.3°. The concentrations of tetracycline were 4.900 mg./L. of hydrochloric acid-potassium chloride buffer or 1.104×10^{-5} *M*. The coincident values are given as half solid circles, ◐.

TABLE III.—ULTRAVIOLET SPECTRAL DATA ON TETRACYCLINES IN ACID SOLUTION

Compound	λ	Absorptivity	λ	Absorptivity	λ	Absorptivity
Tetracycline hydrochloride, standard	218.5	29.23	270	39.52	356	29.85
Tetracycline phosphate, dried	217	26.03	269	33.82	356	25.31
Tetracycline phosphate, non-dried	218	23.49	270	30.74	356	27.34
Heat-degraded tetracycline phosphate, non-dried	218	34.0	270	50.0	356	13.5
Anhydrotetracycline	224	65.73	274	127.82	...	...

tion in 156 hours, 25% in 413 hours, and 50% in 990 hours at 30°. In one day, in 0.01 *M* hydrochloric acid there will be 1.8% decomposition and in 2 days, 3.5% decomposition. At 0°, on the assumption of a heat of activation of 15 Kg. cal./mol., approximately 1% decomposition will occur within a week's time at pH 2.0. The above information should be important in the keeping of standard solutions.

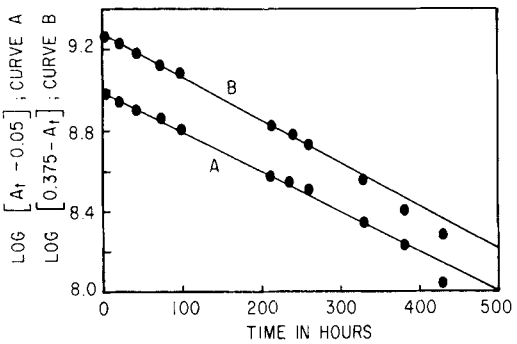


Fig. 4.—First-order rate plots for the acid catalyzed degradation of 1.104×10^{-5} *M* tetracycline hydrochloride in hydrochloric acid-potassium chloride buffer at pH 1.2 at 30°. Curve *A* represents the disappearance of the 356 mμ chromophore; curve *B* represents the appearance of the 270 mμ chromophore. The abscissa is the logarithm of difference of the asymptotic absorbance, A_∞, and the absorbance at any time, A_t.

DISCUSSION

The heat degradation of bulk tetracycline salts occurs when the material contains moisture. However, the normal moisture content of bulk tetracycline salts should not significantly affect the stability at normal temperatures of storage. The dried phosphate and hydrochloride salts of tetracycline are stable at elevated temperatures. Both phosphate and hydrochloride salts of tetracycline degrade at the same rates in acidic solution. This indicates that both salts provide the same tetracycline ion in the acid of the stomach. Thus, no rationale comparable to the greater resistance to acid degradation of penicillin V over G can be proposed for tetracycline phosphate over the hydrochloride.

The validity of the cylinder-plate assay using *B. subtilis* for tetracycline purity is suspect The cylinder-plate assay depends on two parameters: (*a*) rate of diffusion of the antimicrobial agent through the agar to bring it in contact with a greater volume of microorganisms, and (*b*) the minimum inhibitory concentration in the agar media to inhibit microbial growth. It is feasible that a compound derived from the one to be assayed may have less potency but greater diffusibility so that the radius of the clear areas about the dose may be larger for the least potent material. The question as to the specificity of other test organisms is open.

The shift in the ultraviolet spectra of tetracycline after heat degradation in the bulk form or in solution is consistent with what would be expected if it were transformed in part to anhydrotetracycline with aromatization of the C ring. Pertinent spectrophotometric data are given in Table III.

It is apparent that moist tetracycline salts are not completely transformed by heat to a probable anhydrotetracycline. In addition to the appearance of CO_2 on heat degradation that indicates decarboxamidation, it can be estimated on the basis of the increase in absorptivities given in Table III that only 23% of the molecules are aromatized.

On the basis of this observation, anhydrotetracycline and desdimethylaminotetracycline were run in the same cylinder-plate assay as the tetracycline. Anhydrotetracycline and desdimethylaminotetracycline showed twice the activity on weight basis as tetracycline in this assay. Also, a 50-50 mixture of tetracycline and anhydrotetracycline showed twice the activity on a weight basis as tetracycline.

This "case in point" confirms the importance of the axiomatic statement that the specificity of a biological assay of a drug is just as important to evaluate as is its reliability.

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Pharmacological Investigation of the Glucoside and Aglucone Isolated from *Caccinia glauca*

By H. R. K. ARORA† and R. B. ARORA‡

A glucoside and its aglucone isolated from *Caccinia glauca* were studied for their pharmacological activity. Both compounds exhibited a diuretic action which appeared to be due to an increase in glomerular filtration. The dose which induced diuresis did not have any other significant effects. Isolated tissue preparations were not affected except by very high concentrations. The compounds were effective by the oral as well as parenteral route and showed a large safety margin.

CACCINIA GLAUCA Savi., commonly known as gaozaban (1, 2), is frequently used in the indigenous system of medicine as a diuretic, in irritation of the bladder, strangury, and in several other conditions. Various uses for this plant have been described by ancient writers (3, 4).[1] Recently, a glucoside, caccinin, and its aglucone, caccinetin, which is the dimethylallyl ester of caffeic acid, have been isolated from it (5). In view of the reputation and extensive use of the drug by practitioners of the indigenous system of medicine, an investigation was undertaken to elucidate the pharmacological properties of the pure compounds.

METHODS AND RESULTS

Extraction and purification was done as reported earlier (5) and the chemical identity of the pure compounds was confirmed.[2] The glucoside was used as 1–5% solution in normal saline and the aglucone, because of its low solubility in water and saline, as a 1–2% solution in 0.5% sodium bicarbonate.

Cardiovascular Actions.—The effects of these compounds on intact circulation were studied on mongrel dogs of both sexes and male albino rats. The dogs weighed 5–8 Kg. and were anesthetized with an i.v. dose of pentobarbital sodium equivalent to 30 mg./Kg. of body weight. The rats weighed 175–250 Gm. and were anesthetized with an i.p. dose of 25% urethane in water equivalent to 0.6–0.8 ml./100 Gm. of body weight. Blood pressure was recorded from the carotoid artery on a smoked drum. Drugs were injected through a cannulated vein. Electrocardiogram, bipolar limb lead II, was recorded in all experiments.

A fall in blood pressure was recorded, both in rats and dogs, during administration of the glucoside and lasted for 1–3 minutes. Following this, the arterial pressure rose in rats but not in dogs (Fig. 1). The duration and extent of the rise depended upon the dose (Table I). An increase in heart rate was noted in rats but not in dogs. Electrocardiogram remained unchanged with doses up to 200 mg./Kg. of body weight on the glucoside and 50 mg./Kg. of body weight of the aglucone.

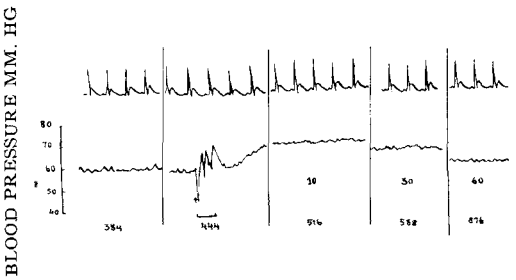


Fig. 1.—From above downwards the records are: electrocardiogram; blood pressure; time in minutes after drug administration; and heart rate per minute showing the effects of the glucoside in the rat. The drug was administered at the time indicated by arrow in a dose of 200 mg./Kg. of body weight intravenously.

The cardiac effects were studied on failing papillary muscle preparation of cat (6), isolated perfused rabbit heart (7), perfused frog ventricle (Straub-Fuehner preparation) (8), and heart-lung preparation of dog (9).

A positive inotropic action was noted on the cat papillary muscle preparation but high concentrations were required to elicit this effect (Table I). Isolated perfused rabbit heart was not significantly affected by doses up to 5 mg. of the glucoside or the aglucone added to the perfusing fluid. Similarly, perfused frog ventricle was unaffected by concentrations of 10^{-4} Gm./ml. of the glucoside or 10^{-3} Gm./ml. of the aglucone, although a concentration of 10^{-3} Gm./ml. of the glucoside reduced heart rate by 45 % ± 9.0 S.E. without producing any other effect.

The heart-lung preparation of dog was unaffected by concentrations of 1.5×10^{-4} Gm./ml. and 6×10^{-4} Gm./ml. of the glucoside. Caffeine, as caffeine citrate, used for comparison, slightly increased venous pressure and decreased competence of the heart when given in a concentration of 1.5×10^{-4} Gm./ml. In a concentration of 6×10^{-4} Gm./ml. it caused an acute fall in blood pressure and a marked rise in venous pressure.

Effect on blood vessels was studied by perfusing rabbit ear vessels (7) with Ringer-Locke solution. Doses of 5 mg. and above of the glucoside or the aglucone reduced the flow through ear vessels by 16 – 20% or more. Lower doses were ineffective.

Received September 29, 1961, from the Department of Pharmacology, Maulana Azad Medical College, New Delhi, India.

Accepted for publication March 6, 1962.

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[1] Original references in Arabic were obtained through the kind courtesy of Hakim Abdul Wahid Saheb of Hamdard Dawakhana, New Delhi, India.

[2] Dr. T. R. Seshadri, Professor of Chemistry, Delhi University, kindly confirmed the chemical identity.

Urinary Actions.—Diuretic activity was studied on conscious and anesthetized rats (10, 11) and anesthetized dogs (12).

In studies on conscious rats, the animals were used in groups of three. All compounds were administered orally. Hourly urine output for the first 5 hours, and the total urine output for the next 19 hours (hereafter referred to as the 24-hr. urine) were noted. The urine outputs of groups receiving test compounds were compared with groups receiving urea or saline only. Chloride and uric acid contents of the total urine excreted during the first 5 hours and the subsequent 19 hours were separately estimated (13, 14).

The glucoside and the aglucone significantly increased the total urine outputs at 5 hours ($0.05 > P > 0.02$) as well as at 24 hours ($0.01 > P > 0.001$) when compared with groups receiving saline only (Table II). The 5- and 24-hr. urine outputs of groups receiving urea were not significantly greater than those receiving the test compounds ($0.5 > P > 0.4$). Chloride excretion was not altered but uric acid excretion was apparently increased. The compounds, however, gave false positive test for uric acid by the method employed.

In anesthetized rats the compounds were injected intravenously and the urine collected every 5 minutes through a polythene catheter placed in the bladder. In this preparation also, urine output was increased by the glucoside as well as the aglucone to the extent of 104% ± 23.8 S.E. and 62% ± 12.8 S.E., respectively. Similarly urine output increased by 85% ± 12.4 S.E. when a dose of 100 mg./Kg. of body weight of the glucoside was injected intravenously in dogs. Chloride excretion in dogs was not affected but endogenous creatinine excretion showed an increase of 92% ± 11.9 S.E. (Table II). Phenol red excretion in dogs was not altered by the glucoside.

Other pharmacological properties.—Intraperitoneal administration of doses up to 400 mg./Kg. of body weight of the glucoside in albino rats failed to

TABLE I.—EFFECT OF GLUCOSIDE AND AGLUCONE ON BLOOD PRESSURE AND HEART RATE OF INTACT ANIMALS AND CAT PAPILLARY MUSCLE[a]

Intact Animals

Animal	No. of Experiments	Drug	Dose, mg./Kg. Body Wt.	Increase, %: Blood Pressure ± S.E.	Increase, %: Heart Rate ± S.E.
Rat	10	Glucoside	50	11.0 ± 2.1	6.0 ± 1.3
Rat	10	Glucoside	100	20.0 ± 2.7	14.0 ± 1.7
Rat	10	Glucoside	200	42.0 ± 2.6	39.0 ± 2.0
Rat	10	Aglucone	100	10.0 ± 1.7	8.0 ± 1.0
Dog	6	Glucoside	100	No rise, only initial fall	8.0 ± 1.9
Dog	6	Aglucone	50	No rise, only initial fall	6.0 ± 2.1

Cat Papillary Muscle

No. of Experiments	Drug	Bath Concentration, Gm./ml.	% Amplitude of Contraction after Drug, ± S.E.
6	Glucoside	10^{-3}	100 ± 11.75
6	Glucoside	2×10^{-4}	64 ± 10.0
6	Glucoside	10^{-4}	44 ± 7.5
6	Aglucone	10^{-3}	88 ± 9.0
6	Aglucone	10^{-4}	46 ± 6.8
6	Caffeine (as citrate)	10^{-3}	98 ± 10.8
6	Caffeine (as citrate)	10^{-4}	58 ± 8.0

[a] Failed to approximately 50% of control amplitude with pentobarbital sodium.
[b] Control amplitude of contraction, prior to inducing failure, was taken as 100 %.
[c] Concentrations are for caffeine base.

TABLE II.—EFFECT OF GLUCOSIDE AND AGLUCONE ON URINE OUTPUT OF RATS AND URINE OUTPUT AND ENDOGENOUS CREATININE EXCRETION OF DOGS

Effect on Rats

Serial No.	Animals, No. Used	Drug	Dose, mg./Kg. Body Wt.	5-hr. Total Urine Output ml./Kg. Body Wt., ± S.E.	24-hr. Total Urine Output, ml./Kg. Body Wt., ± S.E.
1	18	Control (saline only)	...	8.8 ± 1.68	15.5 ± 2.12
2	18	Urea	750	17.0 ± 1.93	22.5 ± 2.54
3	24	Glucoside	50	13.0 ± 1.56	23.0 ± 1.86
4	24	Aglucone	50	12.2 ± 1.70	21.0 ± 2.18

Effect on Dogs

Serial No.	Animals, No. Used	Drug	Dose, mg./Kg. Body Wt.	% Increase in Urine Output after Drug, ± S.E.	% Increase in Endogenous Creatinine Excretion after Drug, ± S.E.
1	6	Glucoside	100	85 ± 12.4	92 ± 11.9

elicit any gross sedation or excitation. Hexobarbital sleeping time in mice (15) was not altered by doses up to 200 mg./Kg. of body weight of the glucoside or 50 mg./Kg. of body weight of the aglucone given intraperitoneally.

No anticonvulsant activity against maximal electroshock and maximal metrazol seizures (16) could be elicited in albino rats with i.p. doses up to 400 mg./Kg. body weight of the glucoside or 50 mg./Kg. body weight of the aglucone. Also, no activity could be detected with these doses against pain induced by application of radiant heat to the rat's tail (17).

A concentration of 10^{-4} Gm./ml. of the glucoside or its aglucone did not have any effect on the movements of isolated rabbit intestine or rat uterus (7). Acetylcholine induced contractions of the isolated rabbit intestine, rat uterus, and dog tracheal muscle preparation (18), were also not influenced by this concentration of the two compounds. A concentration of 5% of the glucoside or 0.5% of the aglucone did not exhibit any mydriatic, miotic, or local anesthetic effect on the rabbit eye. Ciliary movements of the frog esophagus (7) and acetylcholine induced contractions of frog rectus abdominis muscle preparation (7) were also not affected by a concentration of 10^{-3} Gm./ml. of the glucoside or its aglucone This concentration failed to show any antibacterial activity against *Staph. aureus*, *E. coli*, *S. typhi*, and *P. vulgaris* by the agar plate method.

Intravenous LD_{50} of the glucoside in mice (19) was found to be 576 mg./Kg. of body weight. Because of its low solubility, only the oral toxicity of the aglucone, as a 10% suspension in 4% acacia, was studied in rats. No mortality could be recorded with doses upto 2 Gm./Kg. of body weight. Chronic administration of 200 mg./Kg. of body weight of the glucoside and 100 mg./Kg. body weight of the aglucone given subcutaneously to mice failed to alter body weight or induce any histological changes in the liver, kidney, heart, spleen, or brain.

DISCUSSION

The most prominent property of the compounds isolated from *Caccinia glauca* is their diuretic activity. This property is exhibited after oral as well as parenteral administration to conscious and anesthetized rats and anesthetized dogs. Activity is manifested in doses which do not have other pharmacological actions. Thus diuretic activity could be elicited with a dose of 50 mg./Kg. of body weight of the compounds while the same dose given intravenously failed to influence blood pressure, heart rate or electrocardiogram. Only very high doses had any significant cardiovascular activity in the intact preparations. Isolated tissues also were affected by very high concentrations only. Since phenol red and chloride excretions were not affected while endogenous creatinine excretion in dogs was increased in proportion to the increase in urine output, it appears that these compounds act by increasing glomerular filtration. This very much resembles the action of xanthines, which have their main value in cases resistant to mercurial diuretics due to a low glomerular filtration (20) and are the only agents available at present which effectively increase glomerular filtration. The xanthines, however, possess undesirable side effects, especially in large doses, on the cardiovascular, gastrointestinal and central nervous systems. On the contrary, the isolated glucoside and the aglucone, even in large doses, do not exhibit any such undesirable actions as shown by this study. They may, therefore, be considered to have some promise for further studies.

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Pharmacology and Toxicology of Scandium Chloride

By THOMAS J. HALEY, N. KOMESU, L. MAVIS, J. CAWTHORNE, and H. C. UPHAM

The pharmacological and toxicological properties of scandium chloride have been investigated. The intraperitoneal and oral LD_{50}'s were 755 mg./Kg. and 4 Gm./Kg., respectively. Studies of chronic toxicity showed no body weight changes occurred in any group except the male rats on a 1% dietary level. The hemograms of all animals were within the normal range. Histopathological examination of the tissues revealed no damage related to the ingestion of scandium chloride. Transient ocular irritation was observed and the chemical produced extensive scar formation when applied to abraded skin. Pharmacological studies indicated that the chemical had a depressant action on all systems studied and produced lethality by respiratory paralysis coupled with cardiovascular collapse. The lethal effects could not be counteracted by atropinization or epinephrine administration.

In his review of the chemistry of the rare earths, Moeller (1) pointed out that these elements had certain properties which would eventually lead to their industrial usage. However, our knowledge of the biological effects of one of this series, scandium, is almost nonexistent. Mines (2) reported that scandium chloride decreased systole and stopped the perfused frog heart ventricle in diastole. Washing gradually restored its contractility. In this action, scandium was weaker than both erbium and lanthanum. Ishiwara (3) found that scandium exerted an inhibiting effect on mouse carcinoma. Chargaff and Green (4) showed that scandium exhibited an antithrombic effect but showed no antithromboplastic effect. Grant and Kern (5) found that scandium chloride had little or no effect on the intact rabbit eye but produced a permanent opacification of the corneal stroma when the epithelial layer of the cornea had been removed. In an effort to increase our knowledge of the biological actions of scandium chloride, we have investigated its pharmacological and toxicological effects.

EXPERIMENTAL METHODS

The intraperitoneal LD_{50} was obtained with 258 male CF-1 mice and the oral LD_{50} with 130 male CF-1 mice. Chronic toxic effects of scandium chloride were determined by including 0.01, 0.1, and 1.0% of the compound in the diet and feeding it over a period of 90 days to three groups of CRW rats. Each group contained six males and six females. Observations were made every 2 weeks of the following: total erythrocytes, total leukocytes, differential cell count, platelets, hemoglobin, hematocrit, and body weight. Upon completion of the study, histopathological examination was made of the heart, lung, liver, kidney, spleen, pancreas, adrenal, and small intestine. The method of Draize, *et al.* (6), was used to study ocular and skin irritation in rabbits and intradermal irritation in guinea pigs. Three rabbits were used in the ocular studies. Each rabbit had one eye exposed to 0.1 ml. of a 1:1 aqueous scandium chloride while the other eye served as the control. Rabbit skin irritation studies used six animals according to the design of Draize, *et al.* (6). Three guinea pigs were used in the intradermal studies; the concentrations of scandium chloride were: 1:10 to $1:10^6$. Effects of the chemical on isolated guinea pig ileal strips bathed in Locke-Ringers solution were studied in a thermostatically regulated 25-ml. bath using the Trendelenburg method (7). Studies were also made on the isolated rabbit ileum in the presence of 2.5 mcg. of acetylcholine. Ten cats of both sexes, weighing 2.72–3.90. Kg. were anesthetized with 0.5 ml./Kg. of Dial-urethane intraperitoneally. A six channel Offner Dynagraph with Statham transducers was used to record carotid arterial pressure, respiration, nictitating membrane contraction, ECG Lead II, femoral arterial pressure, and femoral arterial flow. The latter was obtained with a 25-ml. Shipley-Wilson flowmeter (8). Preganglionic stimulation of the cervical sympathetic fibers and the contralateral vagus fibers was accomplished with a Grass model S-4 stimulator at 8V/10 sec. Two hours were allowed to elapse prior to beginning drug administration. Intravenous doses of the drugs used were: scandium chloride 0.5 to 20 mg./Kg.; epinephrine 5 mcg./Kg.; acetylcholine 5 mcg./Kg.; histamine 2 mcg./Kg.; and atropine 2 mg./Kg. All injections were made at a constant volume of 1 ml. Where appropriate, the results were analyzed statistically by the Litchfield-Wilcoxon method (9) or standard errors were calculated.

RESULTS

Acute Toxicity.—The symptoms of acute toxicity were immediate defecation, abdominal stretching, depressed respiration, tremor of the hind legs, and sedation. The first deaths occurred within 24 hours but the peak was not reached until 96 hours.

Received March 19, 1962, from the Laboratory of Nuclear Medicine and Radiation Biology of the Department of Biophysics and Nuclear Medicine, School of Medicine, University of California, Los Angeles.

Accepted for publication May 16, 1962.

These studies were supported by Contract AT(04-1)GEN-12 between the Atomic Energy Commission and the University of California.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

Throughout the entire 7-day period of observation the animals were unthrifty. The intraperitoneal LD_{50}/7 days for scandium chloride was 755(741.7–768.6) mg./Kg. with a slope value of 1.06(0.99–1.13). The oral LD_{50}/7 days was 4.0(3.96–4.04) Gm./Kg. with a slope value of 1.03(1.0–1.06).

Chronic Toxicity.—Throughout the 12-week period of feeding the various levels of scandium chloride in the diet, the test animals did not appear to differ in general appearance from the controls. The growth curves (Fig. 1) indicate that, at the dietary levels of scandium chloride studied, there was no significant effect on growth except at the 1% level in male rats. However, at the conclusion of the experimental period even these animals were beginning to approach the control animals in growth. The hemogram of all the animals (Table I) indicated that the chemical had no significant effects on the hematological response of the animals. The changes in the various cells and hemoglobin were probably related to the normal growth pattern of the rats because they were within the ranges given for the rat by Gardner (10). Data on differential counts are not included in Table I because there were no significant differences between the control and treated groups and the data were equivalent to the normal distribution given by Gardner (10). At autopsy, the internal organs of all groups appeared normal; there were no outward signs of damage attributable to the ingestion of scandium chloride for 12 weeks. Furthermore, histopathological examination of the heart, lung, liver, kidney, spleen, pancreas, adrenal, and small intestine revealed no changes which could be related to the ingestion of scandium chloride at the dietary levels studied.

Ocular Irritation.—Direct application of 0.1 ml. of 1:1 scandium chloride solution to the eyes of three rabbits produced a slight translucency in-

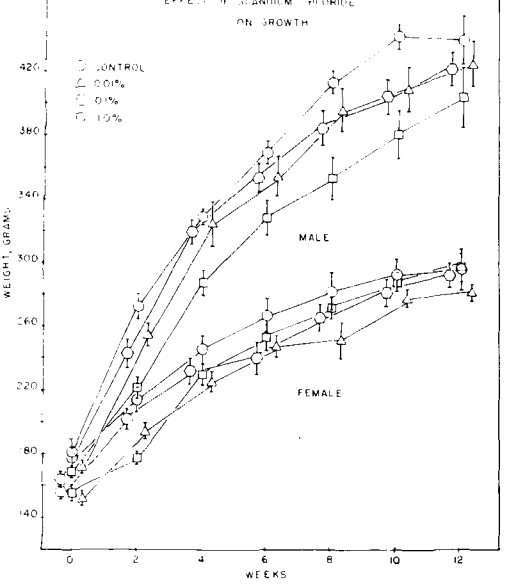


Fig. 1.—Response of male and female CRW rats to feeding various levels of scandium chloride in the diet for 90 days. Bars signify the standard errors. Each group contained six animals for each dosage level.

TABLE I.—HEMATOLOGICAL EFFECTS OF SCANDIUM CHLORIDE

Week		Erythrocytes, mm.^{3} × 10^6 0	Erythrocytes, mm.^{3} × 10^6 12	Leukocytes, mm.^{3} × 10^3 0	Leukocytes, mm.^{3} × 10^3 12	Hematocrit, Vol. % 0	Hematocrit, Vol. % 12	Hemoglobin, Gm. % 0	Hemoglobin, Gm. % 12	Platelets, mm.^{3} × 10^5 0	Platelets, mm.^{3} × 10^5 12
Control	♂ M ± SE[a]	6.38 ± 0.33	9.41 ± 0.20	16.88 ± 0.82	17.73 ± 1.92	41 ± 0.89	50 ± 1.00	12.1 ± 0.31	15.3 ± 0.35	7.35 ± 0.92	6.55 ± 0.37
	Range	4.72 – 7.92	8.70 – 10.50	12.9 – 20.0	11.2 – 26.7	39 – 46	47 – 56	11.3 – 13.5	13.7 – 16.9	4.96 – 11.20	5.52 – 8.88
	♀ M ± SE	7.10 ± 0.46	7.39 ± 0.43	15.44 ± 1.44	15.44 ± 1.69	43 ± 1.26	48 ± 0.71	12.7 ± 0.28	14.0 ± 0.26	6.99 ± 0.67	5.98 ± 0.35
	Range	5.92 – 9.46	5.35 – 8.75	10.2 – 20.5	8.5 – 21.9	38 – 50	45 – 51	11.4 – 13.8	12.8 – 15.1	4.84 – 9.68	4.88 – 7.44
Scandium	♂ M ± SE	6.71 ± 0.31	9.44 ± 0.38	15.42 ± 1.72	15.72 ± 1.97	41 ± 0.80	51 ± 0.56	12.5 ± 0.40	15.1 ± 0.15	7.37 ± 0.62	6.92 ± 0.44
0.01%	Range	6.04 – 7.78	8.29 – 10.42	11.5 – 22.4	10.2 – 22.6	39 – 44	49 – 53	11.4 – 14.1	14.5 – 15.6	5.20 – 9.28	6.16 – 9.04
	♀ M ± SE	6.69 ± 0.16	8.31 ± 0.44	16.23 ± 2.38	17.02 ± 3.06	44 ± 0.79	48 ± 1.17	12.0 ± 0.20	14.5 ± 0.38	8.12 ± 0.55	7.21 ± 0.39
	Range	6.22 – 7.14	6.97 – 9.72	8.9 – 24.8	9.1 – 28.3	41 – 46	45 – 53	11.4 – 12.5	13.5 – 16.1	6.28 – 9.44	5.36 – 8.0
Scandium	♂ M ± SE	6.27 ± 0.38	8.52 ± 0.38	12.15 ± 1.65	16.57 ± 0.74	41 ± 0.72	49 ± 0.67	11.2 ± 0.32	14.7 ± 0.34	7.34 ± 0.82	7.33 ± 0.41
0.1%	Range	5.18 – 7.78	7.50 – 10.0	7.7 – 18.7	14.3 – 19.5	38 – 43	47 – 51	10.2 – 12.1	13.7 – 16.1	4.84 – 10.52	6.40 – 8.88
	♀ M ± SE	6.41 ± 0.49	8.0 ± 0.30	15.67 ± 2.36	12.30 ± 0.97	42 ± 0.67	49 ± 0.31	12.0 ± 0.21	14.9 ± 0.24	8.70 ± 1.09	7.53 ± 0.31
	Range	4.76 – 8.02	6.96 – 8.93	10.2 – 24.1	8.9 – 14.7	40 – 44	48 – 50	11.5 – 13.0	14.2 – 15.6	5.92 – 12.04	6.56 – 8.40
Scandium	♂ M ± SE	5.14 ± 0.29	9.61 ± 0.55	15.68 ± 1.63	14.60 ± 0.82	41 ± 1.21	50 ± 0.62	11.4 ± 0.44	14.5 ± 0.35	8.20 ± 0.74	7.49 ± 0.19
1.0%	Range	4.26 – 6.22	7.15 – 11.05	11.1 – 21.1	12.0 – 16.9	38 – 45	48 – 52	10.0 – 12.7	13.6 – 15.7	6.44 – 11.40	6.96 – 8.16
	♀ M ± SE	6.14 ± 0.51	8.10 ± 0.24	14.23 ± 1.50	12.10 ± 1.19	41 ± 0.75	49 ± 1.03	11.4 ± 0.22	14.2 ± 0.24	7.49 ± 0.59	7.60 ± 0.16
	Range	4.22 – 7.78	7.60 – 9.21	9.4 – 19.6	7.5 – 15.0	39 – 44	46 – 51	10.7 – 12.3	13.7 – 15.3	5.56 – 9.0	7.12 – 8.24

[a] M ± SE = mean ± standard error—based upon six animals per group per dosage level. Based on determinations done every two weeks.

volving less than half of the cornea with a loss of reaction to light by the iris. This corneal reaction completely cleared up within 48 hours and no residual damage was observed. The 24-hour irritation indices for the cornea and iris were 20 and 5, respectively, indicating a transient medium degree of irritation to these structures. Within 1 hour, the conjunctiva was a diffuse beefy red, chemosis had produced a swelling of the eyelids which kept them more than half closed, and there was a discharge which moistened the lids and a considerable area around the eyes. The irritation index reached the maximum score of 20 and small ulcers appeared on the conjunctiva within 24 hours. Healing progressed satisfactorily. All evidence of damage was absent after 96 hours.

Skin Irritation.—Direct application of 0.5 Gm. of crystalline scandium chloride to intact rabbit skin produced no reaction throughout the entire 14-day observation period. There was a very severe reaction on abraded rabbit skin with a maximum irritation index of 8 at 24 hours. No changes in irritation index occurred up to 14 days. However, healing with scar formation was observed at 45 days. The scars were 25 to 30 mm. in diameter. Intradermal injection of scandium chloride at concentrations of 1:10 and 1:100 produced necrosis within 1 hour and an irritation index (erythema plus edema) of 8 which persisted for 7 days. Healing occurred at 14 days with epilation and scar formation in the injected area. At the concentration of 1:1000, the only difference in response was the absence of the early necrosis. The concentrations of $1:10^4$ to $1:10^6$ gave an irritation index of 2 at 24 hours, and complete healing w.thout scar formation occurred at 72 hours.

Effects on Isolated Ileum.—Scandium chloride, dosage range of 10 to 30 mg., produced an increasing depression of intestinal tonus and contractility terminating in complete paralysis of the rabbit ileum. Repeated washing of the five ileal strips did not restore contractility. This depressant effect counteracted the spasmogenic action of acetylcholine. The antispasmodic ED_{50} of scandium chloride was 15.5(8.0–30.2) mg. with a slope value of 2.15(0.65–7.1). Such depressant action also occurred with the Trendelenburg guinea pig preparation where the ED_{50} figures for blocking both the circular and longitudinal muscular contractions, respectively, were: 11.7(8.8–15.3) mg. and 5.6(2.8–11.2) mg. The muscular contractions of this preparation are related to pressure stimulation of the enteric ganglia, thus it would seem that scandium has ganglionic blocking properties. However, experiments with the superior cervical ganglion preparation of the cat indicated that this was unlikely.

Pharmacological Effect.—Within the dosage range of 0.5 to 2 mg./Kg., scandium chloride produced no observable pharmacological effects in the cat. At a dose of 5 mg./Kg. there was a transient loss of blood pressure varying from 15 to 70 mm. Hg and a permanent loss of 10 to 20 mm. Hg from the predrug level. A similar reaction occurred with femoral arterial pressure and blood flow decreased slightly. At this dose the electrocardiogram showed a decrease in the height of the entire complex followed by a high takeoff of the P-wave and a return to normal rhythm and size. In four of the five animals, complete cardiovascular collapse occurred at 10 mg./Kg. and was followed by respiratory paralysis. A similar effect was observed in the other animal at 20 mg./Kg. Prior to exitus, the respiratory rate was not affected. The terminal electrocardiographic changes included a decrease in the entire complex, P-wave absent, T-wave inverted, transient ventricular fibrillation, increased interval between the QRS and the T-wave, a 2 to 1 heart block, inverted QRS complex, and an increased T-wave. Within the dosage range investigated, scandium chloride did not affect the pharmacological responses to acetylcholine, epinephrine, histamine, or vagal stimulation. Furthermore, it had no effect on transmission in the superior cervical ganglion or on contraction of the nictitating membrane. None of the effects of scandium chloride could be counteracted or modified by atropine and the cardiovascular collapse could not be counteracted by epinephrine.

DISCUSSION

From a toxicological viewpoint scandium chloride is less toxic acutely or chronically than gadolinium or samarium (11) or niobium (12) or hafnium (13). However, its detrimental effects on ocular tissues and skin are only exceeded by niobium and hafnium (12, 13). The depressant effects observed by Mines (2) on the isolated frog heart appear to be quite similar to those we observed on the cat heart *in situ*. In general the overall pharmacological effects of scandium chloride on the various body systems studied were of a depressant nature. The mode of death, respiratory paralysis coupled with cardiovascular collapse, was almost identical with what has been observed for the other chemicals in the rare earth series (11–13). Upon the basis of the present investigation, it would appear that scandium chloride exerts its greatest toxicity locally, and proper application of good industrial hygiene practices; respirators, protective clothing, etc.; would prevent acute or chronic exposure.

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Effects of Ionizing Radiation on Two Gelatin Fractions I

Material Preparation, Dosimetry, and Acid-Base Behavior

By LEONARD P. PRUSAK† and BARTLEY J. SCIARRONE

Two fractions, F-I and F-II, were isolated from commercial pigskin gelatin by low-temperature alcohol precipitation. Both fractions, in the form of 1% and 5% solutions and as films, were exposed to variable electron beam irradiation emanating from a 3 Mev Van de Graaff accelerator to give absorbed-dose values of from about 0.1 to about 3 Megarads. Radiation dose was determined by means of Ce(IV)-Ce(III) and polyvinylchloride dosimetry. Acid-base titration curves on the commercial gelatin sample, control fractions F-I and F-II, and irradiated F-I and F-II, as 1% and 5% solutions and as films, all showed differences in —COOH and —NH_2 content.

ASIDE FROM its application to sterilization techniques, the development of ionizing radiation as a research tool has facilitated a more profound understanding of the structure of complex proteins and polymers. Radiation caused by charged and uncharged particles and electromagnetic waves has been widely used by chemists to determine structural vulnerability to high-energy impingement through bond rupture, free radical formation, crosslinking, decarboxylation, and deamination.

Interaction of high-energy radiation with proteins is known to produce a variety of chemical and physical effects, depending on the system. Gelatin was chosen as the test material to study these effects. The wide pharmaceutical importance of gelatin is evidenced by its uninterrupted inclusion in the United States Pharmacopeia since the eighth decennial revision in 1905.

Despite intensive investigation in numerous laboratories, a full explanation of the peculiar behavior of gelatin under varying environment is still unavailable. Interpretative difficulties arise because the material known as "Gelatin" is a heterodisperse system, some fractions having molecular weights as high as 400,000. The distribution of smaller protein molecules depends upon the source and treatment of its precursor, collagen, as well as its subsequent refinement. In general, therefore, more meaningful experimental results are obtainable only after appropriate fractionation, especially when dealing with commercial gelatin.

It is not known to what extent hydrolysis of the protein may occur during separation, such as treatment with alcohol, nor is it known how radiation affects the isoelectric point of the protein. This necessitates the characterization of each fraction by the determination of the milliequivalents of acid and base bound per gram of protein as a function of pH. Hence, these determinations are also useful in comparing the behavior of gelatin fractions following exposure to high-energy radiation at identical levels. This is particularly true if any one or combinations of the following four modes of interaction are operative during irradiation: (*a*) end-group hydrolysis, (*b*) polymerization, (*c*) scission, and (*d*) crosslinking phenomena.

It was intended to establish by this investigation experimental procedures to study the effects of the exposure of gelatin to high-energy radiation. Since we chose to work with commercial pigskin gelatin, a modification of a known fractionation procedure was used to reduce heterogeneity. The modified procedure gave a relatively large molecular weight fraction and a smaller molecular weight fraction both having greater sensitivity and viscosity characteristics (1). There appeared to be no justification to include, in work of this scope, a study of radiation effects on each of several gelatin fractions.

The dosimetry was established using both cericcerous and polyvinylchloride techniques The radiation doses were limited to those producing visually nondiscernible denaturation of the gelatin since acid-base titrations or other physical measurements on solutions containing precipitated protein would not yield readily interpretable results.

Received December 13, 1961, from Rutgers · The State University, College of Pharmacy, Newark, N. J.

Accepted for publication May 14, 1962.

Portion of dissertation submitted to the Graduate School, Rutgers · The State University, by L. P. Prusak in partial fulfillment of the requirements for the Doctor of Philosophy degree.

The authors gratefully acknowledge the consultative aid of Dr. Charles Artandi, Associate Director of Research, Ethicon, Inc., Somerville, N. J., in the performance of radiation experiments.

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Of particular interest was the effect of ionizing radiation on gelatin in the presence and absence of water. For this reason, measurements of the two fractions were attempted on diluted solutions (1%), gels (5%), and solid state films.

EXPERIMENTAL

Material Preparation.—The method of Hultin, *et al.* (1), was modified as follows: Two hundred grams of purified pigskin gelatin (Eastman Kodak Lot No. 5247) were soaked in 4500 ml. distilled water overnight at 25°. The temperature was then increased to 42° whereupon the gelatin immediately dissolved. A sufficient volume of ethanol was then added to bring the volume to 52% v/v, and the mixture was allowed to stand for 24 hours at room temperature. Alcohol was again added to bring the volume to 61%. After 24 hours standing at room temperature, the mixture was halved in volume. The alcohol content of the first half was increased to 72%. After 24 hours, the temperature of the mixture was reduced to −2° and the alcohol content increased to 76%. The mixture was allowed to stand for 72 hours. Ammonium hydroxide was added dropwise to an apparent pH 10, whereupon a precipitate formed which was allowed to settle overnight to permit decantation of the supernate the following morning, leaving Fraction I (F-I).

The second half of the separated volume was allowed to age 4 days at room temperature. The alcohol concentration was then increased to 78%, the temperature reduced to −2°, and ammonium hydroxide added to an apparent pH of 10. A white precipitate formed which was allowed to settle overnight. The supernate was decanted, leaving Fraction II (F-II).

Both fractions (F-I and F-II) were dried under vacuum at room temperature, dialyzed against water and aliquotes lyophilized. Freeze-drying *in vacuo* of F-I produced a dense, hard, white mass which was reduced to granular form. F-II, also white, was characteristically different in texture, less dense, and softer than F-I. Both fractions were stored over Drierite. Yields were 33.2% and 22.4% of F-I and F-II, respectively.

Dosimetry.—Initial experiments on solid-gelatin films and aqueous systems contained in glass were conducted at relatively high (2.5–25 Megarads) ranges on a 6-Mev linear accelerator. All samples suffered degradation, as evidenced by gas evolution and various states of separation from turbidity to heavy precipitation. According to Weiss (2), the radial dimension of glass vessels is critical because of secondary electrons emanating from the vessel wall. As an alternative, polyethylene containers were tried and found to be more satisfactory than glass but precluded irradiating oxygen-free solutions.

Successful irradiation was achieved by exposing the aqueous gelatin systems in 4.5 in. x 6.5 in. heat-sealed polyethylene containers, 3.5 mils average thickness, under the scanned beam of the 3-Mev Van de Graaff accelerator for smaller dosages. Preliminary experimentation on 1% solutions and 5% gels of F-I and F-II indicated upper tolerances of energy at about 2.5–3.0 Megarads for F-I and about 2.0 Megarads for F-II to avoid visible denaturation. When these dose limits for F-I and F-II were exceeded, both the 1% solutions and the 5% gels showed evidence of cloudiness. The 5% gel also became extremely viscous. The degree of apparent denaturation appeared to be proportional to the excessive dose. Therefore, in order to remain below the irradiation dose limits, the Van de Graaff input microamperage was arbitrarily set at 50, 150, 450, and 1000 μ amps. at constant belt speed of 70 in./-min. to give four dose levels with the maximum at about 3 Megarads. (See Table I.)

TABLE I.—COMPARISON OF PVC AND CERIC DOSIMETRY

	Dose (Mrads.) per Individual Run		Average Dose, Mrads.	
	PVC	*Ceric*	*PVC*	*Ceric*
50 μ amps.	0.1	0.1704		
	0.1	0.1588		
	0.1	0.1745	0.12	0.16
	0.1	0.1110		
	0.2	0.2080		
150 μ amps.	0.3	0.4364		
	0.4	0.4709		
	0.3	0.3984	0.38	0.45
	0.4	0.4440		
	0.5	0.5337		
450 μ amps.	1.0	1.2710		
	1.0	1.1890		
	1.0	1.3570	1.10	1.31
	1.2	1.3240		
	1.3	1.4400		
10^3 μ amps.	2.8	2.803		
	2.1	2.558		
	2.5	3.020	2.60	2.87
	2.8	2.949		
	2.9	3.020		

Exposure to the electron beam was carried out by placing one sealed polyethylene unit containing 20 ml. of a 1% solution, a second unit containing 20 ml. of a 5% solution and two film strips of a given fraction on 9 in. x 15 in. aluminum trays provided with $^3/_4$ in. wood backing to minimize back scattering. All physical measurements were made on freshly irradiated gelatin solutions and films.

In addition to the gelatin solutions and films to be irradiated, each tray included a polyethylene unit containing 20 ml. of freshly prepared 0.015 *M* ceric ammonium sulfate in 0.8 *N* sulfuric acid (in triple distilled water) for precise dosimetry calculations and one rigid polyvinylchloride (PVC) slide for additional dosage approximation. Weiss (2) found that ceric dosimetry is very reliable when the total absorbed dose is in the order of 6 million rep. Subsequently Harlan, *et al.* (3), established the reliability of ceric dosimetry for high level irradiation measurements up to 10^8 rads. In our hands the ceric system was accurate and reproducible within the dose range applied in the experiments.

Immediately prior to irradiation a 0.005 *M* standard ferrous ammonium sulfate solution was prepared. This was used to standardize the nonirradiated ceric ammonium sulfate and to back-titrate the irradiated volumes, using 1,10-phenanthroline as the end-point indicator. Irradiation runs were completed on the same day and titrations were made immediately following exposure to minimize error due to air-induced oxidation. From the original concentration data and the back-titration, the number of micro-

moles of Ce(IV) reduced (or Ce(III) produced) was calculated. The total absorbed dose, E_t in units of *ev/L.*, was determined from ceric dosimetry through the following (3, 4)

$$E_t = (6.02 \times 10^{17}) \times (\mu \text{ moles Ce(IV) reduced}) \times \left[\frac{1000}{\text{Vol. used}}\right] \times \left[\frac{100}{2.5}\right]$$

and converted to Megarads using the relationship

$$6.37 \times 10^{22} \text{ ev/L.} = 1 \text{ Megarad.}$$

Rigid polyvinylchloride (PVC) dosimetry, as described by Artandi, *et al.* (5), was used concurrently with the ceric indicator. Although the sensitivity of PVC at low levels does not approach that of the ceric-cerous system, its application in the present work as an approximation for absorbed energy proved highly desirable. Table I shows PVC and ceric determinations on a series of five irradiation runs. These data were analyzed and the best fitting equation was calculated to be

$$Y = 0.9259X - 0.0575, 0.11 \leq X \leq 3.02$$

where, Y = PVC reading and X = ceric reading. The above equation is valid only over the stated range of X. The degree of fit, or how well the Y values depended on the X values, was 98.5%, which is considered good.

Acid-Base Behavior.—All titrations were conducted under a nitrogen atmosphere on 10-ml. solutions which were 1% with respect to the gelatin concentration. Several drops of octyl alcohol were added as an antifoaming agent. Titrant volumes of 0.1 *N* sodium hydroxide and 0.1 *N* hydrochloric acid were measured with a model SB2 syringe microburet. The pH values were measured with a Beckman model G pH meter employing a glass and saturated calomel electrode system.

Titrations were performed on the commercial gelatin, irradiated and unirradiated water controls, and fractions F-I and F-II, the latter two as (*a*) controls, (*b*) irradiated 1% solutions, (*c*) irradiated 5% gels, and (*d*) irradiated films. Titration volumes were corrected by subtracting the appropriate water controls. The titration curves were obtained by plotting the observed pH (abscissa) against the milliequivalents of acid or base bound per gram of protein. Gelatin concentrations for this purpose were obtained by dry weight.

In order to provide comparison among the three forms of each fraction, as well as between the two fractions, four-level irradiation was conducted on all forms of both fractions simultaneously. The average dose imparted to each of four separate passes under the electron beam was calculated to be: (*a*) 0.37, (*b*) 0.84, (*c*) 2.00, and (*d*) 3.00 Megarads, respectively.

RESULTS AND DISCUSSION

Unirradiated F-I and F-II titration curves, although nearly identical and characterized by equilibrium pH 7.0, represented a distinct shift from their parent source which showed a pH of 4.8 at equilibrium. (See Fig. 1.) Irradiated F-I and F-II titration curves showed nearly complete superimposability for a given form at various irradiation levels. Thus F-I, irradiated as the 1% solution,

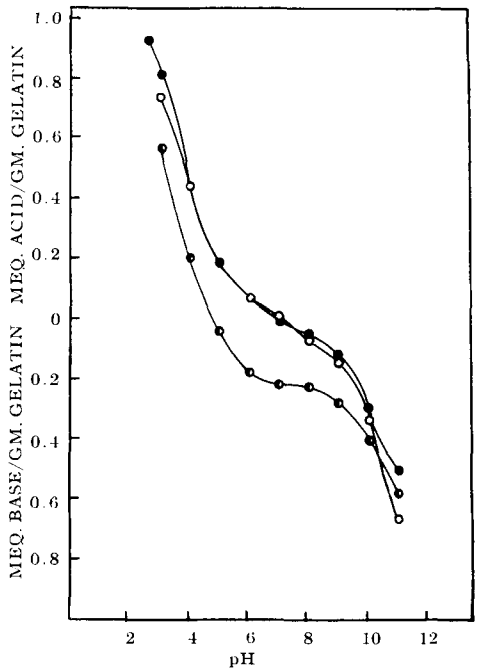


Fig. 1.—Titration curves of controls in the form of unirradiated F-I (○), F-II (●), and commercial gelatin (◐).

showed four nearly identical curves at 0.37; 0.84; 2.00; and 3.00 Mrads. The same pattern prevailed in the remaining five forms and was especially pronounced in the case of irradiated films, with slight departures at the extreme ends of the curves.

Comparison of F-I and F-II at any given radiation level with respect to a particular form, *e.g.*, 1% solution, showed differences in structure. Figure 2 is illustrative. The 1% solutions irradiated at 0.37 Mrad. appeared to be similar at either end of the titration range, but in the region from pH 4.0 to 10.0 more acid than base volumes were required to bind F-I than F-II. Values fell at pH 5.5 and pH 7.0, respectively. At the 0.847 Mrad. level, solutions of both fractions at the same concentration showed similar patterns in the extreme acid and base regions. Deviation in milliequivalents of acid/base bound per gram of protein began at pH 3.0, increased gradually to a maximum at pH 6.0–8.0, then returned slowly to near similarity. Although the equilibrium pH of F-I did not shift (5.5) despite the twofold increase in absorbed dose, a slight increase appeared in F-II (pH 7.2).

By increasing the irradiation dose to 2.0 Mrads., titration curves on 1% solutions of F-I and F-II continued to show the inversely proportional relationship between dose and milliequivalents of acid/base bound in the midportion of the curve. When the irradiation dose was further increased to 3.0 Mrads., curves of F-I and F-II approached superimposability, with only a small difference in milliequivalent values in the midportion.

Plots of F-I and F-II 5% solutions differed from their 1% counterparts along the entire titration curve. A consistently gradual approach toward pH 7.0 in the four cases studied from either extreme was apparent. Figure 3 is illustrative of this series. More milliequivalents of acid were required to bind

1 Gm. of F-I than F-II. This differential was gradually reduced until pH 7.0 where no difference existed and the curves crossed. F-II showed progressively greater milliequivalent requirements and increasing departure from F-I as the extreme alkaline end of the curve was approached.

Films of F-I and F-II, at all four irradiation levels studied, showed remarkably consistent similarity in titration curve behavior. Figure 4 shows a representative curve of a film of F-I irradiated at 0.37 Mrad. in this series. Slight variations (*ca.* 0.02 milliequivalents) among the various plots in isolated sections of the curves were not considered critical, all eight plots being substantially similar within limits of experimental error. Equilibrium values for films of F-I and F-II at all four irradiation doses consistently fell at pH 7.0.

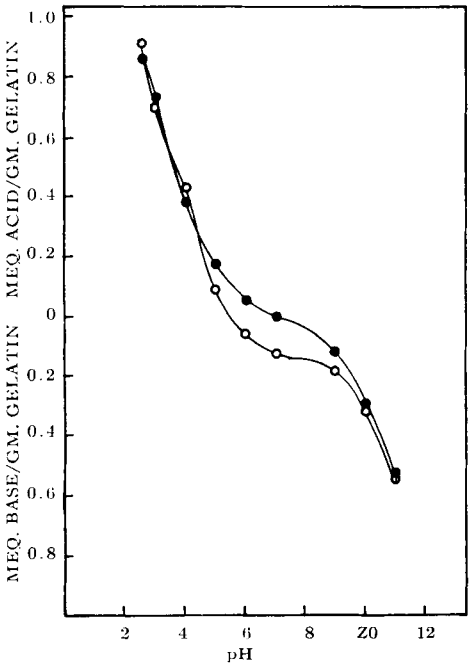


Fig. 2.—Titration curves of F-I (○) and F-II (●) after 1% dispersions of each were subjected to irradiation dose 0.37 of Mrads.

CONCLUSIONS

Treatment of commercial gelatin (pH 4.9) with alcohol under low-temperature conditions and adjustment of pH to a value on the alkaline side appears to disrupt the charge on the parent gelatin molecule, reducing its acid/base binding capacity and shifting the pH to neutrality. In changing from one form to another, there is a loss of amide nitrogen in the form of ammonia, resulting in an increase in the number of free carboxyl groups and base-fixing capacity (6). Further, the shift in position of the titration curve can be attributed to the liberation of carboxyl groups concurrent with release of amine ammonia. The shift is the same regardless of molecular weight

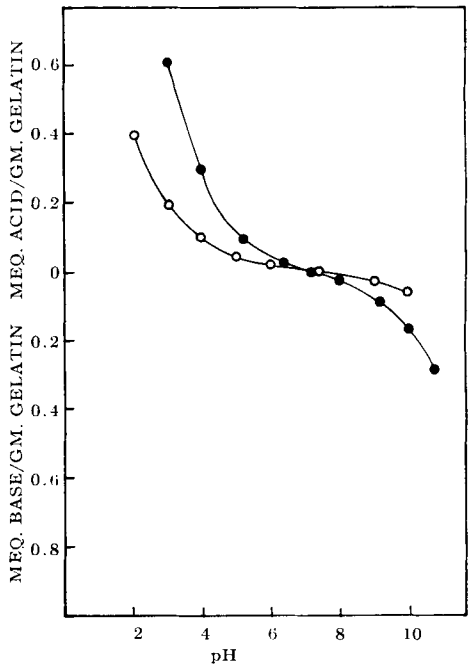


Fig. 3.—Titration curves of F-I (○) and F-II (●) after 5% dispersions of each were subjected to irradiation dose of 20 Mrads.

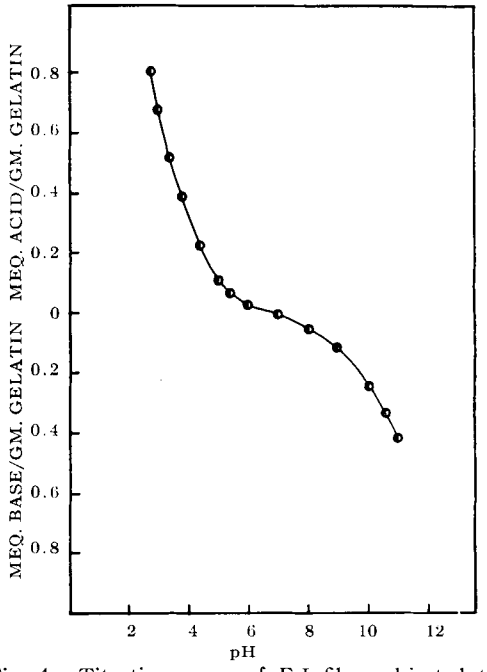


Fig. 4.—Titration curve of F-I film subjected to irradiation dose of 0.37 Mrads.

characteristics of the fractionated components.

Irradiation at similar levels of the two gelatin fractions as 1% solutions does not materially affect either their acid-binding or base-binding capacity at the distal ends of the titration curves.

In the region of neutrality, and 2 pH units on either side of it, a substantial variance occurs at the lowest irradiation level which gradually lessens as the dose is increased. To this portion of the curve are attributable the imidazole groups of the protein. It is evident that the low molecular weight fraction (F-II) requires acid/-base milliequivalents of constant value in this region whereas the high molecular weight fraction initially (0.37 Megarads) binds 0.12 milliequivalents of base; this requirement gradually diminishes as the dose of ionizing radiation is increased.

In 1:20 solute:solvent ratio during the ionization process, a given gelatin fraction shows entirely different charge structure from that when irradiation occurs at 1:100 ratio. The decrease in volume requirements of acid and base indicates a reduced availability of titratable amino and carboxyl groups.

In subsequent reports we shall present the results of further studies showing experimental evidence supporting our belief that the two fractions not only differed from each other but behaved differently under changing irradiation stress.

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Kinetics of Steroid Effects on Ca^{47} Dynamics in Dogs with the Analog Computer I

By EDWARD R. GARRETT†, RICHARD L. JOHNSTON, and ELLIOTT J. COLLINS

Intravenous administration of the short-lived isotope Ca^{47} in dogs was followed by the measurement of Ca^{47} in blood, feces, and urine during a control phase, a steroid regimen (2 mg./Kg./day), and a final recovery, post-steroid phase. Methods and procedures were developed to analyze the data by a combination of digital and analog computer techniques. In all dogs and in all phases the specific rate constants of a polyexponential fit for the distribution of Ca^{47} in all hypothetical compartments under all the experimental conditions studied were similar. The cumulative fecal and urinary Ca^{47} eliminations were fitted to a first order curve as a function of time by the analog computer. The most striking effect of steroid therapy in the young dog used as his own control is the increase in first order Ca^{47} elimination within the first 200 hours to $1\frac{1}{2}$ to 2 times the control values. After cessation of steroid therapy, the per cent of the total intravenous dose eliminated decreased and approached the amount of the control period within these 200 hours. The residual Ca^{47} is tied up in the bone and is slowly released at an apparently linear rate over longer periods of time. The rate of release between 200 and 700 hours is twice as great with steroid regimen as without for both control and recovery phases.

RADIOCALCIUM and other bone-seeking isotopes have been used in kinetic analyses of changes in blood and excretion of injected radioisotope (1–4). The radioisotope, Ca^{47}, has recently become available and presents certain advantages over previous radiocalcium studies in that it is a gamma emitter of short half-life of 4.56 days and an animal can be used as his own control in short-term sequential studies of the effect of a therapeutic agent or experimental condition on the kinetics of radiocalcium distribution, metabolism, and excretion.

The advantages of this isotope have permitted us to initiate an investigation of the osteoporotic effects of adrenal steroid regimen and its potential reversibility. Preliminary studies have demonstrated that this phenomenon is common to all the adrenal steroids in varying degree (5, 6) but one was chosen for the detailed study presented in this paper.

This paper presents a detailed quantitative analysis of our first studies on the effects of adrenal steroids on the distribution and kinetics of intravenously administered Ca^{47}. The digital- and analog-computer techniques described herein greatly facilitated the quantification of the huge masses of data it was necessary to obtain in such studies.

Special emphasis was also placed on the initial disappearance curves of radiocalcium from the blood, studies of which have not received too much attention previously (7).

Received March 9, 1962, from the Research Division, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication March 15, 1962.
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METHODS

Treatment of Animals.—Three beagle dogs and one mongrel were used in these experiments. The animals were conditioned to the metabolism cages and laboratory routine prior to experimentation. Pertinent data for dogs 0 through 3, given as initial age in months, body weight in Kg. and sex are for dog 0: 7, 9.30, M; dog 1: 7, 7.50, M; dog 2: 9, 6.25, F; and dog 3: mongrel of indeterminate age, 9.20, F. The methods and procedures of intravenous administration of Ca^{47} and of the determination of radioactivity in blood serum, urine, and feces have been detailed elsewhere (5).

The studies were conducted in five sequential phases. Ten days after the initial administration and study of the distribution of 50 μc. of Ca^{47} (control, study *A*), 2 mg./Kg./day of 6α-methylprednisolone were given orally. Two weeks later, Ca^{47} was again administered and the distribution studied (steroid, study *B*). Five weeks after initiating steroid therapy, Ca^{47} was again administered and the distribution studied (steroid, study *C*). Steroid therapy was reduced at the rate of 1 mg./day for 52 days and was halted 112 days after its inception. The radioisotope Ca^{47} was administered and the distribution studied at 40 days (recovery, study *D*) and at 117 days (recovery, study *E*) after cessation of steroid therapy.

The dogs were sampled for blood after intravenous administration of Ca^{47} at 2, 4, 6, 8, 10, and 15 minutes; then at 15-minute intervals to 120 minutes; at 30-minute intervals to 4 hours; at hourly intervals to 9 hours; at 12, 24, 30, 36, and 48 hours; and at subsequent 24-hour intervals for 10 days.

The urine was collected at 4, 8, 12, 24, 30, 36, and 48 hours and at subsequent daily intervals until the study was terminated. Catheterization was used throughout the first 48 hours. The feces were collected at the end of 12 hours and at subsequent daily intervals.

In the studies biopsies were performed on a long bone and costal bone 48 hours after radioisotope administration, each time from a different limb. Antibiotics, tetracycline phosphate and novobiocin, were administered for 8 days after surgery. The site of the biopsy was replicated as closely as possible. However, the data were so variable from dog to dog and without any consistent pattern among the sequence of studies *A* through *E* that they are not worth reporting. Similar experiences have been encountered from biopsies of bone after radioisotope administration by Rich (8).

Treatment of Data.—The actual counts of Ca^{47} in blood, feces, and urine were converted into the % of the initial count of the dose of Ca^{47} given to each dog. This necessitated the counting of a standard aliquot of Ca^{47} in solution simultaneously with the samples of the studies to correct the values for the natural radioactive decay of Ca^{47}. The errors in counting were computed by statistical methods considering the time of counting and the magnitude of the count for the tissue, the standard, and the background (9).

The blood serum data reported consisted of the time of blood withdrawal, the total radiation count, c for v ml. of serum counted for t minutes. Alternately, a background count c_B, was taken for t_B minutes. A fraction of the Ca^{47} solution used for injection was conserved as a standard and was counted alternately with all samples, a count of c_S for t_S minutes. The initial total Ca^{47} dose, D in c.p.m. after correction for background, was also counted alternately with the standard, c_{S_o} minutes, at the time of intravenous administration of the dose. The Burroughs *E*-102 digital computer was programed to calculate and read out the Ca^{47} content of blood serum at time t, $Ca_B{}^{47}$, per ml. of serum as the % of the initial total dose as

$$Ca_B{}^{47} \text{ (per ml. serum, \% of total dose)} = 100\, q(c/tv - c_B/t_B)/D \quad \text{(Eq. 1)}$$

where

$$q = (c_{S_o}/t_o - c_{B_o}/t_o)/(c_S/t_S - c_B/t_B) = S_o/S \quad \text{(Eq. 2)}$$

The error E due to counting per ml. serum as the % of the initial total dose can be estimated from

$$E = Ca_B{}^{47}\sigma_{qc}/qc \quad \text{(Eq. 3)}$$

where the counting error of D is ignored since D is a constant for all serum samples. The fractional error in the count after correction for the normal radioactive decay of Ca^{47} can be estimated (10) as

$$\sigma_{qc}/qc = \sqrt{\sigma_c{}^2/c^2 + \sigma_q{}^2/q^2} \quad \text{(Eq. 4)}$$

where

$$\sigma_c = K\sqrt{c/t^2 + c_B/t_B{}^2} \quad \text{(Eq. 5)}$$

and

$$\sigma_q = q\sqrt{\sigma_{S_o}{}^2/S_o{}^2 + \sigma_S{}^2/S^2} \quad \text{(Eq. 6)}$$

$$\sigma_S = K\sqrt{c_S/t_S{}^2 + c_B/t_B{}^2} \quad \text{(Eq. 7)}$$

$$\sigma_{S_o} = K\sqrt{c_{S_o}/t_{S_o}{}^2 + c_B/t_B{}^2} \quad \text{(Eq. 8)}$$

where the K values are given in the literature (9).

When 9.158×10^6 c.p.m. of Ca^{47} were intravenously administered with a standard count at the time of injection of $S_o = 3891$ for $t_{S_o} = 1$ minute with a background count of $c_B = 1070$ for $t_B = 30$ minutes, typical data for a blood serum sample taken 2 minutes later are: $c = 6625$, $t = 1$ minute, $v = 1$ ml., $c_B/t_B = 36$ c.p.m., $c_S = 3868$ for $t_S = 1$ minute and thus $q = 1.006263$, and $Ca_B{}^{47} = 0.0724\%$ of initial radioactive dose per ml. of serum. With a K of 1.96 for 95% limits (9) $\sigma_S = 122.4647$, $\sigma_{S_o} = 122.28968$, $\sigma_c = 159.9653$, then $\sigma_q = 0.045305$, $\sigma_{qc} = 340.42131$, $\sigma_{qc}/qc = 0.051064$, and the error E due to counting variability is $0.0724 \pm 0.0037\%$.

The urine and feces data reported were similar to that of the blood except that the volume of urine collected V was included with the v ml. of urine counted c for t minutes; and the weight of feces collected W was included with w g of feces counted c for t minutes. The methods of calculation for Ca^{47} excreted by feces and urine were similar to those outlined for the blood serum except that the fecal and urine excretion of Ca^{47} was calculated in terms of the % of the total dose

$$P = 100q(c/t - c_B/t)W/wD \quad \text{(Eq. 9)}$$

where W/w of the feces becomes V/v in the case of urine.

The cumulative % excretion of injected dose at any time is summed, $\sum_{i=1}^{n} P_i$, and the error E in the accumulated ΣP_i is estimated from

$$E(\Sigma P_i) = \sqrt{\sum_{i=1}^{n} \sigma_i^2} \qquad \text{(Eq. 10)}$$

where a σ_i for a particular i^{th} urine or fecal sample is equivalent to the previously described σ_{qc}.

For a typical urine sample taken 4 hours after intravenous injection of Ca^{47}, $c = 5467$ for $v = 4.0$ ml. counted for $t = 5$ minutes with a background count of $c_B = 1385$ for 30 minutes and a standard count of $c_S = 10773$ for $t_S = 1$ minute. The total volume of urine, $V = 5.5$ ml. The correction factor calculated out to $q = 1.06704$ so that the % of the initial dose of Ca^{47} excreted in this urine sample was $P = 0.02685\%$. Since this was the first urine sample, $\Sigma P_i = P_1$. The error computations were $\sigma_S = 203.4489$, $\sigma_{S_0} = 210.1275$, $\sigma_q = 0.028165$, $\sigma_c = 29.08595$, $\sigma_{qc} = 43.6772$, so that the fractional error of the count was $\sigma_{qc}/qc = 0.03744$. The error in % of the original dose excreted in the urine was $0.0268 \pm 0.0010\%$.

For a typical fecal sample taken 24 hours after intravenous injection of Ca^{47}, $c = 879$ for $w = 2.8437$ g counted for $t = 1.0$ minute with a background count of $c_B/t_B = 33$ c.p.m. The total weight of the feces was $W = 730$ g. The correction factor calculated out to $q = 1.318133$ so that the % of the initial dose of Ca^{47} excreted in this fecal sample was $P = 4.8356$. The error computations were $\sigma_S = 208.2955$, $\sigma_{S_0} = 238.7188$, $\sigma_q = 0.03240$, $\sigma_c = 59.1907$, $\sigma_{qc} = 83.0112$ so that the fractional error of the corrected count was $\sigma_{qc}/qc = 0.07165$. The error E in % of the original dose excreted in this fecal sample was 4.84 ± 0.351.

Analog Computer Programing and Operation for Serum Data.—The Ca_B^{47} content of a ml. of the blood serum expressed as the % of the total radioactive dose is a function of time and can be described by an additive series of exponentials (1, 7, 11–13)

$$Ca_B^{47} = \sum_{i=1}^{n} A_i e^{-k_i t} \qquad \text{(Eq. 11)}$$

The final exponential of the series, $e^{-k_n t}$, can be obtained by a direct analog computer fitting, *i.e.*, by determining the best potentiometer setting, k_n, and best initial voltage A_n to fit the terminal data of a plot of Ca_B^{47} against time by the discharge of a capacitor (14, 15).

An equivalent procedure which gives the same information as to k_n and A_n is to plot the logarithm of Ca_B^{47} against time and determine the k_n from the slope ($= k_n/2.303$) and the A_n from the intercept (log A_n at $t = 0$) of the semilogarithmic plot for the terminal data of the blood serum Ca^{47} as a function of time. Typical plots are given in Fig. 1 for the data obtained in excess of 1,000 minutes after Ca^{47} administration.

The values plotted as log Ca_B^{47} between 1,500 and 7,000 minutes (Fig. 1) approximate linearity and provide a good estimate of a first order decrease of radioisotope for the elimination of Ca^{47} from the blood characterized by an exponential. The data beyond 7,000 minutes were frequently higher than could be expected on the premise that this was the true terminal exponential. However, these aberrant values would have little effect on the summation of exponential expressions

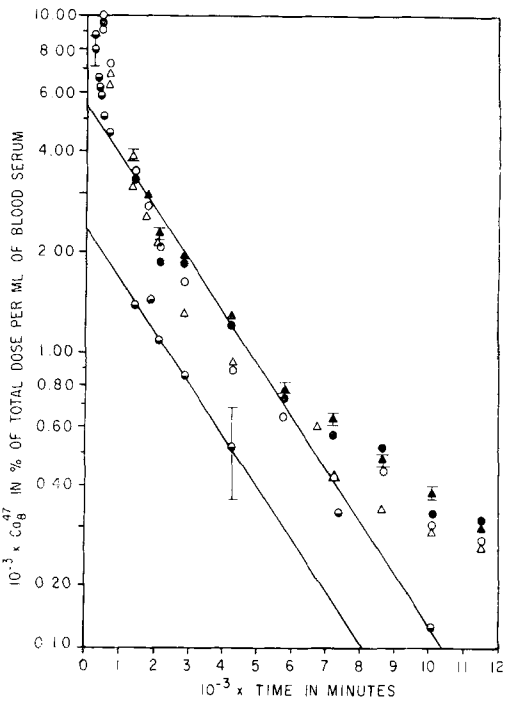


Fig. 1.—Typical semi-logarithmic plots of Ca_B^{47} in blood, % of total dose per ml. of serum, against time for that portion of total curve assigned to the final exponential. The data are for dog 0, studies A, ◐; B, ⊙; C, ●; D, △; and E, ▲. The horizontal lines above and below some of the data indicate the 95% confidence limits due to counting error.

$$\sum_{i=1}^{n-1} A_i e^{-k_i t} = Ca_B^{47} - A_n e^{-k_n t} \qquad \text{(Eq. 12)}$$

where Ca_B^{47} was defined in Eq. 11.

Both the analog computer and graphical techniques described gave similar values of k_n and A_n.

The analog computer program for determining the sum of exponentials is given in Fig. 2. The data calculated from Eq. 12 were fitted by the read out on the Mosely model 14 recorder summed from the necessary exponential functions so as to fully describe the overall decrease of Ca^{47} with time. The rapid decrease of Ca^{47} content of blood serum in the first 15 minutes was independent of the slower decreases over the remaining first thousand minutes. This is fitted on the analog computer with the best A_1 and k_1 value. Subsequently, an A_2 and k_2 value was attempted to account for the remaining data. It was found that two more exponentials were necessary to fully characterize the data so that the data have been plotted as $Ca_B^{47} - A_4 e^{-k_4 t}$ and is equal to $A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t}$. The $A_2 + A_3$ values and k_2 and k_3 values of the potentiometers were chosen to completely fit the data.

Thus the expression

$$Ca_B^{47} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t} + A_4 e^{-k_4 t} \qquad \text{(Eq. 13)}$$

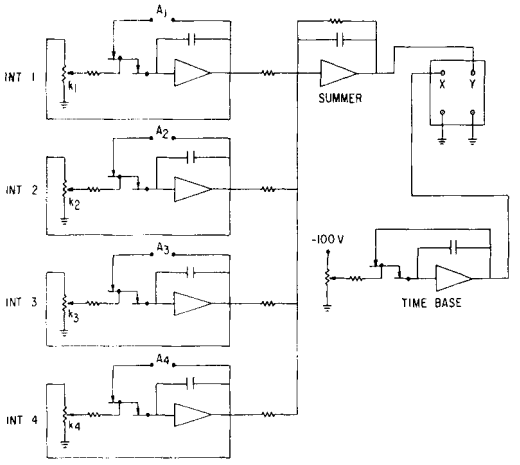


Fig. 2.—Analog computer program for fitting the sum of exponentials to plots of Ca_B^{47} in blood, % of total dose per ml. of serum, against time.

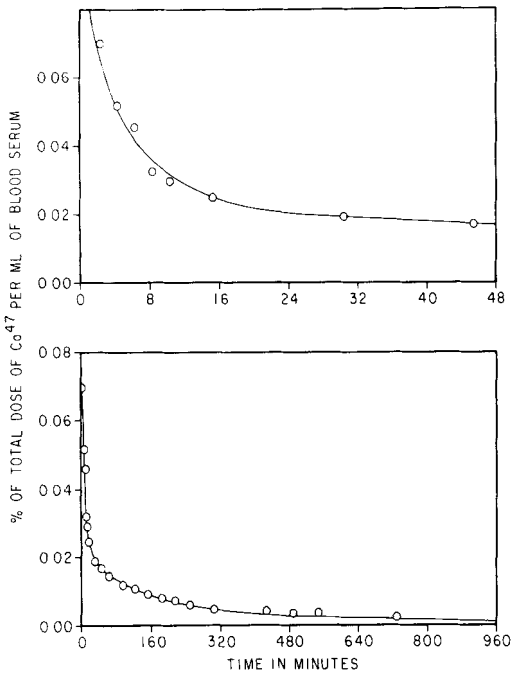


Fig. 3.—Examples of goodness of fit of analog computation of summed polyexponentials to the early phases of the plots of Ca_B^{47} in blood, % of total dose of Ca^{47} per ml. of blood serum against time, dog O, study *E*.

and the determined A_i and k_i values accounted for all the Ca_B^{47} data as a function of time with the possible exception of amounts in the order of thousandths of the original dose in the blood in excess of 7,000 minutes after administration of the dose. The drawn lines in Figs. 3 and 4 are typical examples of the goodness of fit obtained from the program of Fig. 2 and plotted out on the recorder. The A_i and k_i data so obtained for all studies on all 4 dogs are given in Table I.

TABLE I.—TABULATION OF CONSTANTS[a] FOR ADDITIVE SERIES OF EXPONENTIAL $A_i e^{-k_i t}$, CHARACTERIZING THE DECREASE OF Ca^{47} FROM THE BLOOD AFTER INTRAVENOUS ADMINISTRATION[b]

Study[c]	Dog O				Dog 1[d]		Dog 1			Dog 2		
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
A_1	0.0203	0.0380	0.0642	0.0527	0.0613	0.0108	0.0958	0.0646	0.0772	0.0766	0.0561	0.0416	0.0133	0.0432	0.0400	0.0363	0.0633
A_2	0.0114	0.0191	0.0188	0.0158	0.0186	0.0232	0.0284	0.0190	0.0200	0.0260	0.0268	0.0193	0.0071	0.0199	0.0197	0.0143	0.0302
A_3	0.0090	0.0119	0.0130	0.0109	0.0141	0.0106	0.0214	0.0192	0.0298	0.0244	0.0158	0.0191	0.0060	0.0176	0.0191	0.0123	0.0167
A_4	0.0024	0.0045	0.0055	0.0049	0.0053	0.0038	0.0060	0.0067	0.0086	0.0076	0.0108	0.0089	0.0063	0.0080	0.0068	0.0113	0.0087
$\Sigma_i A_i$	0.0431	0.0735	0.1015	0.0845	0.0993	0.0484	0.1516	0.1095	0.1356	0.1546	0.1095	0.0889	0.0827	0.0887	0.0856	0.0742	0.1189
$\Sigma_i A_i - A_1$	0.0288	0.0255	0.0373	0.0316	0.0380	0.0376	0.0558	0.0449	0.0584	0.0580	0.0534	0.0473	0.0194	0.0455	0.0456	0.0379	0.0556
$10^3 k_1$	4.48	4.17	4.17	2.75	4.64	4.19	6.37	3.30	4.20	4.11	2.94	4.39	3.49	4.02	4.04	2.80	4.53
$10^4 k_2$	1.37	1.38	2.47	2.21	5.56	2.10	3.62	3.40	3.42	3.77	3.11	6.70	3.27	3.37	3.02	3.10	2.91
$10^5 k_3$	3.24	3.19	3.19	3.15	4.41	2.96	3.25	3.23	3.22	3.22	2.48	4.05	5.90	2.74	2.63	3.07	3.50
$10^6 k_4$	5.90	5.45	5.45	6.06	6.30	5.90	5.50	5.74	5.90	5.45	5.90	5.19	5.90	5.90	5.90	5.50	5.06

[a] A_i are in % of initial total dose of Ca^{47}/ml. of serum and k_i are in sec.$^{-1}$. [b] Study *A* was the control study. Studies *B* and *C* were the steroid administration studies. Studies *D* and *E* were the recovery studies, *i.e.*, after cessation of steroid administration. [c] The injected doses of Ca^{47} were, in c.p.m., 5.92×10^6 for *A*, 9.16×10^6 for *B*, 15.2×10^6 for *C*, 5.72×10^6 for *D*, and 8.62×10^6 for *E*. The only exception was *A*, Dog 3, *viz.*, 2.96×10^6 c.p.m. [d] Dog deceased after this run.

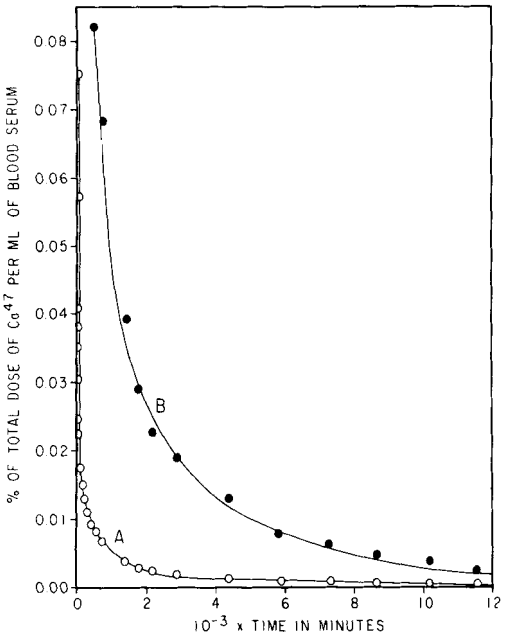


Fig. 4.—Examples of goodness of fit of analog computation of summed polyexponentials to the later phases of the plots of Ca_B^{47} in blood, % of total dose of Ca^{47} per ml. of blood serum against time, dog O, study *E*. Curve B is a ten-fold magnification, and the ordinate values should be divided by 10.

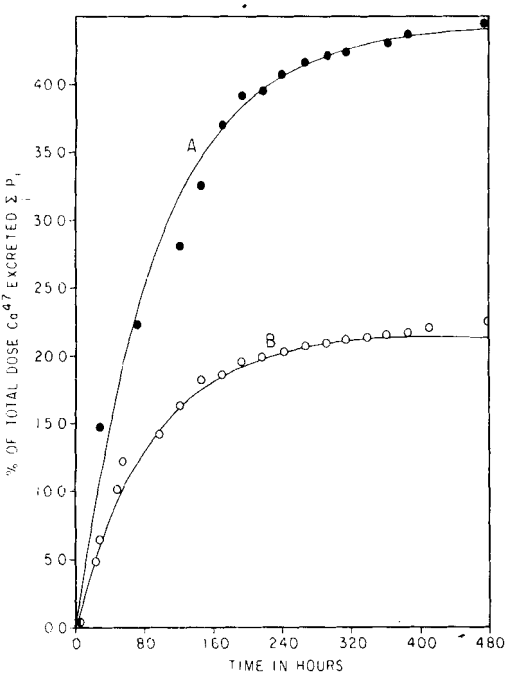


Fig. 5.—Examples of goodness of fit of analog computation of pseudo first-order fecal excretion with time, $\Sigma P_i = P_\infty (1 - e^{-kt})$, of cumulative Ca^{47}. Curve A is for dog 2, study *E*; Curve B is for dog O, study *E*.

Feces and Urine Data.—The Ca^{47} appearing in the urine and feces is cumulatively plotted as ΣP_i against time as in the typical curves of Figs. 5 and 6 for feces and is reasonably consistent with the expression

$$\Sigma P_i = P_\infty(1 - e^{-kt}) \qquad \text{(Eq. 14)}$$

where P_∞ is the total % of the total dose of Ca^{47} appearing in the feces (or urine) over the entire period on the postulate of first-order elimination as defined in Eq. 14.

The Ca^{47} data from feces (or urine) were fitted to this expression on the assumption that none had been eliminated at zero time. It was observed that the first-order fit was not strictly true, that an increase in the ΣP_i, percentage of total Ca^{47} dose excreted in urine and feces, occurs for a longer period of time than that interval defined by the blood studies, that fecal and urinary elimination is not strictly first-order in that a slight positive deviation from the first-order asymptote occurs with time, Fig. 6. Also, a short lag period before the initial fecal elimination of Ca^{47} is to be expected due to gastrointestinal holdup and was indicated in several of the studies. The urinary elimination mirrors the fecal elimination and a set of typical cumulative curves for all studies on one dog is given in Fig. 7. The drawn lines are typical examples of the goodness of analog computer fitting to the first-order expression of Eq. 14.

RESULTS

Blood serum.—The A_i and k_i values for the sum of exponentials in Eq. 13 that describe the Ca_B^{47} content of blood serum as a function of time are given in Table I for the various dogs and studies.

Typical analog computer fittings of the data from which these values were derived are given in Figs. 3 and 4. In general, the fit was very good by a four-factor exponential except perhaps for the very tail end of the curve in excess of 7,000 minutes where the data maintained a level above zero. The counting error is included in the size of the data circles.

Inspection of Table I shows that there is no great difference in the k_i values for any of the four exponentials of Eq. 13 whether the dog is under control (study *A*), under steroid regimen (studies *B* and *C*), or in recovery from steroid regimen (studies *D* and *E*). All the stated k_i values for any given factor are within the error of the fit regardless of the experimental condition of the animal.

The major difference within dogs and among the studies under the various experimental conditions was in the magnitude of the A_i values or the total amount of Ca^{47} transferred from the blood as characterized by the four exponentials.

This is most readily and graphically demonstrated in the example plotted in Fig. 8 where the log Ca_B^{47} *vs.* time for all studies in a given dog are parallel (*i.e.*, the k_i are similar) but the curves are displaced along the ordinate (*i.e.*, the amounts of Ca_B^{47} appearing in the blood vary with the experimental condition of the animal.).

This can be observed with the $\Sigma_i A_i$ values given in Table I and indicates that animals under steroid therapy (studies *B* and *C*) have higher Ca^{47} contents

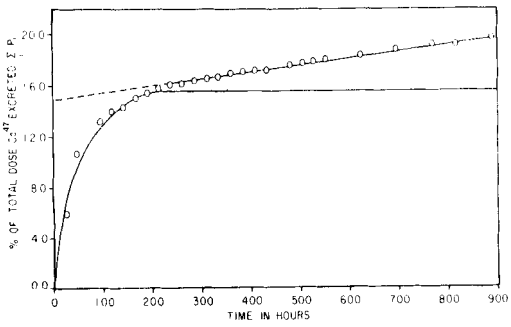


Fig. 6.—Example of zero order deviation $\Sigma P_i = mt + b$, from the analog computer fitted pseudo first-order asymptote P_∞, of the cumulative fecal excretion with time, dog O, study *D*.

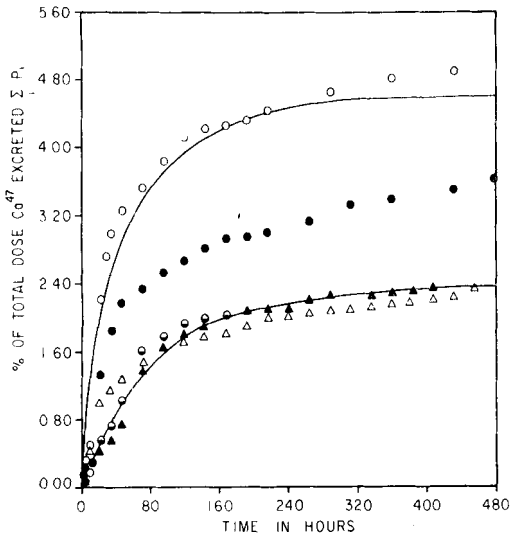


Fig. 7.—Typical examples of cumulative urinary and fecal excretion of Ca^{47} with time for a dog under different experimental conditions with typical analog computer fits. The urinary Ca^{47} excretion of dog 2; study *A*, ◐; *B*, ⊙; *C*, ●; *D*, ◬; and *E*, ▲.

in the blood than control animals (study *A*) or on recovery from steroid (studies *D* and *E*). The only study that provides an exception from this latter case is study *E* of dog 3.

With all dogs the A_1 and A_2 values of the control (study *A*) were less than that value for the steroid regimen (studies *B* and *C*).

Feces and urine.—The P_∞ and k values in accordance with the first order cumulative excretion of Ca^{47} as in Eq. 14 are given in Table II as fitted to the plots of total Ca^{47} excreted in feces (or urine) *vs.* time by the analog computer with the curve forced through the origin. Typical plots of such fits are given in Figs. 5 and 6 for feces and in Fig. 7 for urine. Since the data are cumulative, large deviations may be expected from a smooth curve with the initial fecal Ca^{47} contents. The good fit is well demonstrated for the duration of time covered by the blood studies. The counting error is included in the circles representing the data.

Over longer periods of study the fecal and urinary elimination of Ca^{47} deviates from first order, and this deviation over the theoretical asymptote, P_∞, can be approximated by a straight line of slight slope (Fig. 6)

$$\Sigma P_i = mt + b \qquad \text{(Eq. 15)}$$

where the slope m in % of total dose excreted per hour and intercept b are also given in Table II.

Inspection of the fecal and urinary elimination curves for all studies and dogs clearly showed that the amount of Ca^{47} appearing in both urine and feces for dogs on steroid regimen is greater than for these dogs during their control period as is demonstrated in the typical plots of Fig. 7. In addition, during the period of recovery from the steroid regimen, significantly less Ca^{47} appeared in the feces and urine.

The rate constants k among runs for a particular dog and among dogs show no significant correlation with the treatments and no highly significant variation outside of the experimental error of their estimates. Thus the k_u for urinary elimination is *ca.* 5×10^{-6} sec.$^{-1}$ ($t_{1/2}$ = 39 hours), and the k_f for fecal elimination is *ca.* 3×10^{-6} sec.$^{-1}$($t_{1/2}$ = 64 hours). The possible gastrointestinal lag in these latter cases may give erroneously low values for k_f.

The deviations defined in Eq. 15 of Ca^{47} data ΣP_i in the urine and feces from the first-order accumulative asymptote P_∞ defined in Eq. 14 are significant with respect to the counting errors. Unfortunately, fecal and urinary data were not accumulated beyond 170 hours for the control run and no deviation from first-order elimination of Ca^{47} could be calculated. Inspection of the data, however, indicates that in the first recovery study *D* on feces, the slopes m are less than for the steroid studies *B* and *C*. For both steroid recovery studies *D* and *E*, on urine, the slopes m are less than for the steroid studies *B* and *C*.

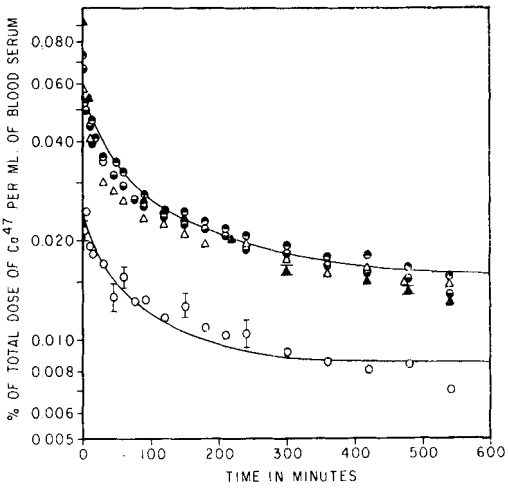


Fig. 8.—Typical examples of Ca_B^{47} blood content with time for the early phases of a dog under different experimental conditions with typical analog computer fits, dog 3, study *A*, ⊙; *B*, ◓; *C*, ◒; *D*, ◬; and *E*, ▲. The horizontal lines above and below some of the data indicate the 95% confidence limits due to counting error.

TABLE II.—CONSTANTS FOR CHARACTERIZATION OF ACCUMULATION OF Ca^{47} IN DOG FECES AND URINE FROM 1,[a] $\Sigma P_i = P_\infty (1 - e^{-kt})$ AND 2[b], $\Sigma P_i = mt + b$

Dog	Study	Feces				Urine			
		P_∞	10^6k	m	b	P_∞	10^6k	m	b
0	A	18.5	2.60	...[d]	...	1.10	5.98	...[d]	...
	B	30.0	4.37	0.0202[e]	24.6	1.14	4.11	0.0015[e]	0.76
	C	33.9	3.28	0.0257[e]	28.8	1.62	4.73	0.0012[e]	1.30
	D	15.5	5.43	0.0054[f]	14.9	0.397	4.32	0.00116[e]	0.21
	E	21.6	3.19	0.108[e]	17.5	0.845	1.92	0.00097[e]	0.45
1	A	21.0	2.97	...[d]	...	1.026	8.38	...[d]	...
	B[c]	31.9	2.56	0.0207[e]	22.5	3.33	5.61	0.00338[e]	2.64
2	A	35.0	3.70	...[d]	...	2.06	5.83	...[d]	...
	B	41.5	5.06	0.0225[e]	36.3	4.40	5.91	0.00162[e]	3.67
	C	35.75	3.30	0.167[e]	30.0	3.09	5.01	0.00182[e]	5.30
	D	27.25	2.06	0.0056[e]	20.2	1.86	5.79	0.00138[e]	1.73
	E	44.4	2.89	0.0170[e]	37.1	2.335	3.18	0.00069[e]	0.61
3	A	55.9	2.66	...[d]	...	2.80	6.09	...[d]	...
	B	61.0	4.62	0.0183[e]	52.7	4.14	4.91	0.00258[e]	3.85
	C	60.9	3.82	0.0173[e]	51.9	5.77	5.12	0.00237[e]	2.52
	D	25.0	1.46	0.0041[f]	21.1	2.025	5.20	0.00135[e]	1.67
	E	30.4	4.14	0.0163[e]	25.6	0.876	2.54	0.00112[e]	1.88

[a] Where ΣP_i is expressed in terms of % of total dose and k in sec.$^{-1}$. [b] This is the later deviation from the first-order accumulation of fecal and urinary Ca^{47}, *i.e.*, from P_∞ where m is in % of total dose/hour and t is in hours. [c] Dog deceased after this run. [d] No values obtained beyond 170 hours after Ca^{47} administration. [e] *Ca.* 200 to *ca.* 450 hours. [f] *Ca.* 370 to *ca.* 900 hours.

DISCUSSION

Blood serum.—The disappearance of labeled Ca^{47} from the blood of the dog after intravenous administration can be represented by a four-factor linear homogeneous equation of exponentials as in Eq. 13 in agreement with the observations of Thomas, *et al.* (2), for the rabbit and Bronner, *et al.* (4), for man.

This mechanistically implies that the initial 100% of the Ca^{47} administered distributes itself into four compartments as

$$\sum_1^4 A_i = A_1 + A_2 + A_3 + A_4 \quad \text{(Eq. 16)}$$

and that a certain fraction, *i.e.*, $A_1/\Sigma_i A_i$, $A_2/\Sigma_i A_i$, etc., leaves the blood into these compartments with respective rate constants k_1, k_2, k_3, and k_4. This also implies that the losses of Ca^{47} into each of these compartments are independent processes. This is not a physiologically true model since the transfer of Ca^{47} from blood into the tissue, although it may simulate a first-order process, actually may be a series of equilibria, or one transfer in a sequence of transfers, and the k_i and A_i may be interdependent.

However, the summation of exponentials of Eq. 13 may serve as a first approximation of a physiological model (2). The major contribution to the first exponential may be the relatively fast diffusion or transfer of Ca^{47} into extravascular spaces, some soft tissues, and the gastrointestinal tract. The major contribution to the second and third exponentials may be the slower diffusion or transfer into other depots. One such may be a metabolic pool, *e.g.*, chelation and/or complexation with bone surface. A simultaneous contribution may be the apparently irreversible incorporation of radioisotope into bone, *i.e.*, Ca^{47} covered by subsequent calcium deposition and unavailable for ready redistribution among blood and tissues during the interval studied. The major contribution to the fourth exponential may be the fecal and urinary elimination of radioisotope where the first-order accumulative rates are of similar magnitude as the fourth exponential. The positive deviations from first-order eliminations and the fourth exponential characterizing the blood curves may be due to resorption of radioisotope from bone. It must be emphasized that these cited assignments are oversimplified possibilities.

From the A_i data of Table I, the apparent volume of a particular compartment in terms of equivalent ml. of serum may be estimated by $1/A_i$ where $1/\Sigma A_i$ is an estimate of the total ml. of serum in the dog. The effect of steroid regimen is to increase the Ca^{47} content of the blood, a phenomenon which is reversed on cessation of such therapy. This may indicate that steroid therapy affects the instantaneous diffusion of Ca^{47} from the blood to some easily accessible other compartment characterized by A_1 and k_1 and/or the blood volume is significantly decreased by steroid therapy. There are too many variables extant in this study to permit a definite assignment of cause. This phenomenon will be further evaluated in future studies.

Urine and feces.—If it is postulated that the Ca^{47} is either in the feces, urine, or some "deep" compartment that may be "bone" at 200–400 hours after administration, then the difference of the measured amounts excreted from the total dose could give the % of the original dose in the "bone." Steroid regimen decreased the incorporation of calcium into the "bone" and with cessation of steroid regimen provided a "rebound" effect, an increased incorporation of blood calcium into "bone." On these premises, the gross effect of adrenal steroid administration is to induce a reversible osteoporosis. The procedures described herein can provide a quantitative measure.

The deviations of the cumulative Ca^{47} excreted in the urine and feces from first-order asymptotes were significant with respect to counting errors. It is naive to assume that the slow diffusion of Ca^{47} through the "bone" from the sites of "accretion"

to the sites of "resorption," the subsequent reentry into the blood and the final elimination into the urine and feces is a simple constant rate that is only apparent after the first-order accumulation of the greater portion of Ca^{47} excreted in feces and urine. However, it may be practical to use the slope of this straight line as a possible measure of "resorption" of calcium from "bone." The quantification of these slopes well indicates a decrease in the rates of "resorption" from bone on cessation of steroid therapy, a possible physiological "rebound" from steroid therapy. The data were insufficient to contrast steroid regimen with the control period.

The male beagles showed greater incorporation of calcium into the "deep" compartment than the slightly older female. The older mongrel showed the least such incorporation but the greatest "rebound."

SUMMARY

Methods and procedures have been developed to utilize the analog computation of a summation of four polyexponentials to describe the Ca^{47} content of blood as a function of time after intravenous administration. Quantitative measures of determining calcium incorporation into a "deep" compartment that may be synonymous with "bone" have been described. The exponential constants for the distribution and elimination of Ca^{47} for all dogs and under all experimental conditions studied, *i.e.*, control, steroid, and recovery periods, were similar.

The short half-life of Ca^{47} of 4.56 days permitted a sequence of studies of experimental effects on calcium dynamics as followed by the study of the distribution and excretion of Ca^{47} with each dog used as his own control.

The most pronounced effect of steroid regimen in dogs is the increased amount of Ca^{47} accumulated in the feces over the control period. A similar effect is observed with the Ca^{47} accumulated in the urine although the percentage of the total administered dose excreted in the urine (2–6%) is very much less than that excreted in the feces (20–60%).

A possible, but less well defined effect of steroid regimen, is the decrease in apparent blood volume from the control period as based on radioisotopic measurement of Ca^{47}-labeled blood. It is also well indicated that the rate of Ca^{47} return from the "deep" compartment is accelerated by steroid therapy. In the classical nomenclature, it may be that bone "accretion" is inhibited and bone "resorption" accelerated by steroid therapy to induce the reversible osteoporosis of Cushing's syndrome.

A "rebound" effect is also noted in that on cessation of steroid therapy, the dog utilizes more Ca^{47} and the rate of return from the "deep" compartment is lessened.

The possible utilization of more effective osteoporotic agents as measured by these techniques should be of great interest in the removal of bone-seeking radioisotopes produced from radiation or fall-out contamination. Also, these methods are applicable to the recognition and quantification of nonosteoporotic anti-inflammatory agents, and therapeutic agents that may prevent or treat bone diseases.

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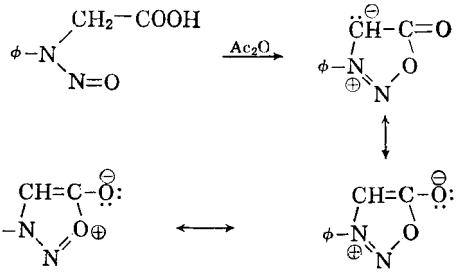
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Synthesis of Sydnones as Potential Therapeutic Agents

By LEMONT B. KIER and DEVINDRA DHAWAN

A number of new 3-alkyl sydnones have been prepared as potential therapeutic agents. A preliminary pharmacological evaluation indicated that these compounds were central nervous system stimulants. The partition coefficients between chloroform and water were determined and a correlation was found between this and the millimole dosage per kilogram in the sydnones with nonfunctional side chains. Ultraviolet and infrared data are reported for the sydnones.

THE FIRST mesoionic compounds known as sydnones (I) were synthesized in 1935 by Earl and Mackney at the University of Sydney (1). They observed that when N-nitroso-N-phenyl glycine is heated with acetic anhydride, water is lost and a compound having a 1,2,3-oxadiazole ring structure is formed:

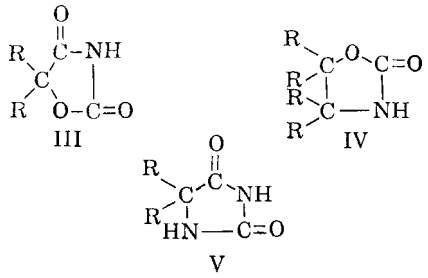


The bicyclic structure (II), originally postulated, was unacceptable to subsequent investigators (2–4) who showed that the sydnones are resonance hybrids of numerous canonical forms, including the three above. Dipole moment (5, 6), X-ray analysis (7), and molecular-orbital treatment (8) have subsequently substantiated this structural assignment. The term "mesoionic" has been coined for this type of system and has been defined (9) as a five- or six-membered heterocyclic compound, which cannot be represented satisfactorily by any one canonical structure. It possesses a sextet of π electrons in association with all of the ring atoms. Further, the ring bears a fractional positive charge balanced by a corresponding negative charge on a covalently attached exocyclic atom or group. Structures like I are used by convention for mesoionic compounds.

A review of the literature revealed no pharma-



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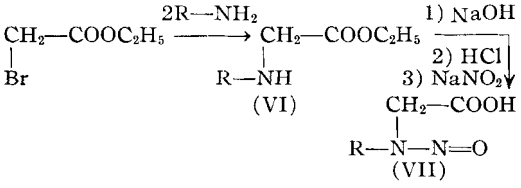


III IV V

cological study of these compounds as possible chemotherapeutic agents.[1] A comparison of the sydnone (I) structure with that of the 2,4-oxazolidinediones (III), 2-oxazolidinone (IV), and hydantoins (V) revealed some similarity. These last three are known anticonvulsants though alkylation of the nitrogen atom will frequently reverse the action, producing a convulsant (10, 11).

With these thoughts in mind, a systematic study of some sydnones and other mesoionic compounds was initiated in this laboratory. For this initial study a number of new 3-substituted sydnones were prepared representing the simplest type of substitution on the ring.

Among several routes leading to the nitrosoamino acid (VII), the following one was chosen



A recent study on the synthesis of α-alkylamino esters (12) of the type VI showed that a higher yield could be obtained by using an α-bromo-ester and two equivalents of the amine. The second equivalent of the amine proved to be an acid scavenger superior to trialkyl amines or inorganic bases. All of the α-alkylamino esters in our study were high-boiling liquids which could be easily purified by vacuum distillation. See

Received March 16, 1962, from the Department of Pharmaceutical Chemistry, University of Florida, College of Pharmacy, Gainesville.

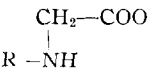
Accepted for publication April 23, 1962.

[1] Since completion of this manuscript, another report covering antitumor activity of several sydnones has been published (23).

Table I. Hydrolysis of VI was done in refluxing base. Isolation and purification of the resulting α-alkylamino acids proved to be very tedious and costly. Since the α-alkylamino esters could be easily purified and characterized, isolation of their hydrolysis products was considered unnecessary. This product was then nitrosated *in situ* after rendering the solution strongly acid. All of the nitrosoamino acids (VII) were solids and were purified with no difficulty. See Table II.

A number of reagents have been employed for the ring closure of VII to the sydnone including acetic anhydride and trifluoroacetic anhydride (4, 13). The mechanism involves the formation of a mixed anhydride with the nitroso acid (VII). Because of the grater lability of the C_4 hydrogen, due to the absence of an alkyl group on the same carbon, it was felt that acetic anhydride would be a satisfactory ring-closing reagent. The exclusion of water in this step was essential for maximum yield, hence freshly distilled acetic anhydride was employed throughout. The initial heating followed by a prolonged period of standing in the dark was found to give the best yield of sydnone (I). The solids were generally crystallized from water while the liquids were vacuum distilled. Several explosions resulted from distilling the allyl sydnone until it was distilled under nitrogen. See Table III for analyses.

TABLE I.—ETHYL ALKYLAMINO ACETATES



R	Formula	% Yield	B.p., °C./ mm.	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
Isopropyl	$C_7H_{15}O_2N$	53	36/10	57.90	10.41	9.65	57.53	10.13	9.36
Propyl	$C_7H_{15}O_2N$	72	40/0.25	57.90	10.41	9.65	57.73	10.44	9.69
Allyl	$C_7H_{13}O_2N$	57	67/20	58.72	9.15	9.73	58.54	8.90	9.87
sec-Butyl	$C_8H_{17}O_2N$	34	57/0.4	60.34	10.76	8.80	60.30	10.84	8.90
tert-Butyl	$C_8H_{17}O_2N$	38	65/3	60.34	10.76	8.80	60.13	10.73	8.63
Furfuryl	$C_9H_{13}O_3N$	36	90/0.1	59.00	7.15	7.65	59.36	7.29	7.33
Phenethyl	$C_{11}H_{15}O_2N$	72	70/0.5	68.37	7.82	7.25	68.62	8.20	6.88
tert-Octyl[a]	$C_{12}H_{25}O_2N$	55	75/22	66.93	11.70	6.51	66.59	11.89	6.53

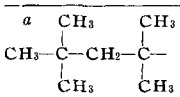
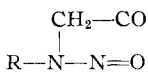
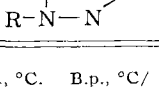
[a] 

TABLE II.—N-NITROSO ALKYLAMINO ACETIC ACIDS



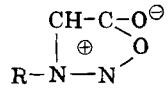
R	Formula	% Yield	M.p., °C.	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
Isopropyl	$C_5H_{10}O_3N_2$	50	129–130	41.09	6.80	19.17	41.25	6.90	18.90
Propyl	$C_5H_{10}O_3N_2$	51	72–74	41.09	6.80	19.17	40.90	6.69	18.96
Allyl	$C_5H_8O_3N_2$	65	20	41.66	5.59	19.44	41.35	5.74	19.75
sec-Butyl	$C_6H_{12}O_3N_2$	65	79–81	44.99	7.55	17.49	44.87	7.26	17.26
tert-Butyl	$C_6H_{12}O_3N_2$	56	109–110	44.99	7.55	17.49	54.29	7.19	17.78
Furfuryl	$C_7H_8O_4N_2$	50	114–115	45.65	4.38	15.21	45.47	4.39	14.91
Phenethyl	$C_{10}H_{12}O_3N_2$	55	134–136	57.68	5.81	13.46	57.50	5.90	13.21
tert-Octyl	$C_{10}H_{20}O_3N_2$	85	135–137	55.53	9.32	12.95	55.22	9.12	12.74

TABLE III.—N-ALKYL SYDNONES



R	Formula	% Yield	M.p., °C.	B.p., °C/ mm.	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
Isopropyl	$C_5H_8N_2O_2$	63	54–55.5	...	46.87	6.29	21.89	47.06	6.38	21.92
Propyl	$C_5H_8N_2O_2$	68	...	94/2.5	46.87	6.29	21.89	46.68	6.36	21.79
Allyl	$C_5H_6N_2O_2$	71	...	70/2	47.62	4.80	22.22	47.78	5.17	22.58
sec-Butyl	$C_6H_{10}N_2O_2$	67	...	160/3	50.70	7.09	19.71	50.85	6.95	19.99
tert-Butyl	$C_6H_{10}N_2O_2$	55	168–170	...	50.70	7.09	19.71	50.53	7.19	19.87
Furfuryl	$C_7H_6N_2O_3$	60	94–95	...	50.60	3.64	16.86	50.48	3.66	16.53
Phenethyl	$C_{10}H_{10}N_2O_2$	52	...	111/5	63.14	5.30	14.73	63.37	5.32	14.53
tert-Octyl	$C_{10}H_{18}N_2O_2$	72	93–95	...	60.58	9.15	14.14	60.39	9.31	14.36

TABLE IV.—SYDNONE PHYSICAL AND PHARMACOLOGICAL DATA



R	Ethyl Alcohol λ max. mμ	ε	C-O, λ Nujol μ	Pc $\frac{C_1HCl_3}{H_2O}$	CD_{50}, mmole/Kg.	LD_{50}, mmole/Kg.
Allyl	290	6000	5.75	0.90	1.11	1.21
Isopropyl	289	6600	5.75	1.3	2.96	3.75
Propyl	288	6600	5.75	1.5	1.60	1.87
Furfuryl	290	6500	5.85	3.4	>2.5	>2.5
tert-Butyl	290	5900	5.80	4.6	0.87	1.30
sec-Butyl	290	6300	5.75	6.4	1.13	1.55
tert-Octyl	292	6100	5.81	9.6	0.25	0.31
Phenethyl	286	5800	5.75	20.	...	>6.0

The ultraviolet absorption maxima fell in the range reported for a few of these compounds (14–16). An infrared maximum at 5.75–5.85 μ was found for each sydnone, which can be ascribed to the pseudo-keto group (17–19). In addition, each sydnone had a strong absorption band at 9.5 μ which is in the region of the C—O stretching frequencies (20).

A preliminary pharmacological evaluation (21) indicated that all but the phenethyl sydnone were central nervous system stimulants with a particularly stimulating effect on respiration. As comparative parameters the LD_{50} and CD_{50} (convulsive dose) i.p. in mice were chosen. See Table IV.

In an attempt to relate structural effect with pharmacological action, the partition coefficients between chloroform and water were determined. Because of the simple nature of the modifying groups it was felt that the ring itself imparted the pharmacological action while the side chain determined the rate of absorption and transport to the receptor site.

If the pharmacological action was chiefly central, as preliminary data indicated, then the sydnones with a greater affinity for lipid solvents would be expected to penetrate the lipid barrier into the CNS with greater facility. This conjecture was proven to be correct as far as the sydnones with nonfunctional aliphatic side chains. Those with a higher partition coefficient between chloroform and water were more active in eliciting convulsions on a millimole per kilogram basis. Those with functional groups in the side chain such as the allyl, phenethyl, and furfuryl sydnones did not follow this pattern. Both of these aspects of mono and disubstituted sydnones are under current study in this laboratory.

EXPERIMENTAL [2]

Alkylamino Esters (VI).—A solution of two moles of the amine in 500 ml. of anhydrous ether was cooled to 0° in a two-necked flask equipped for stirring. To this was added dropwise, one mole of ethyl bromoacetate in 150 ml. of anhydrous ether. The temperature was maintained at 0°. The mixture was stirred for 3 hours then allowed to stand overnight. The precipitate was filtered off and the filtrate vacuum distilled. In all cases the products were liquids. See Table I for yield, b.p., and analyses. Infrared analyses corroborated the assigned structures.

Hydrolysis of Esters.—The alkylamino esters (VI) were hydrolyzed by refluxing them with 1.5 equivalents of aqueous sodium hydroxide. The refluxing was stopped when the reaction mixture formed a single phase. The solution was then cooled and made acid to pH 2 with hydrochloric acid.

Nitrosoamino Acids (VII).—The strongly acidified solution of the alkylamino acid was cooled to 0° in an ice-salt bath and then treated dropwise with a solution containing 1.1 equivalents of sodium nitrite. This reaction mixture was stirred for two hours at 0° after the addition. The crude nitrosoamino acid was filtered and recrystallized from ether, acetone, or mixtures of the two. All of the nitrosoamino acids gave positive Liebermann tests (22). See Table II for yields, m.p., and analyses.

3-Alkyl Sydnones (I).—The nitrosoamino acids (VII) were added to a five molar excess of freshly distilled acetic anhydride and warmed on a steam bath for 3 hours. The reaction mixture was allowed to stand for three days at room temperature in the dark. The acetic anhydride was removed under vacuum using toluene to remove the last traces. The sydnone was then either distilled under vacuum or crystallized from ether. Distillation of the 3-allyl sydnone had to be done under nitrogen in order to prevent the ignition or explosion of the product. See Table III for m.p. or b.p., yield, and analyses. The infrared and ultraviolet spectra were obtained for each compound. See Table IV.

Partition Coefficients.—The partition coefficients of the sydnones (I) were obtained between water-saturated chloroform and chloroform-saturated water. Several concentrations of each compound in water-saturated chloroform at 23° were shaken for 1 hour with varying quantities of chloroform-saturated water. The concentration in the chloroform before and after shaking with the water was determined from the ultraviolet absorption at the maximum. An average of at least four determinations for each sydnone was used. See Table IV.

[2] All melting points were done on the Kofler apparatus and are corrected. Analyses were performed by Weiler and Strauss Microanalytical Labs., Oxford, England and Galbraith Laboratories, Knoxville, Tenn.

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Protective Coatings XIII. Amphoteric Polyvinylpyridine Derivatives

By TADAO IDA, SHUZO KISHI, SHOJI TAKAHASHI, and ISAMU UTSUMI

Polyampholites of vinylpyridine or its alkyl derivatives with acrylic or methacrylic acid and the copolymers of these compounds with other vinyl derivatives were synthesized and studied as protective coating agents for tablets. Results showed that the synthesized copolymers had adequate viscoelasticity, protective qualities, and disintegration characteristics to serve as protective coating agents.

POLYAMPHOLYTES of vinylpyridine or its derivatives with acrylic or methacrylic acid and copolymers of those with other vinyl compounds were experimentally synthesized. The purpose of synthesis was to obtain such protective-coating agents as to exhibit protective activity on drugs and solubility in both gastric and intestinal juice. Solubility, water vapor permeability through the film, and viscoelasticity were tested on each of copolymers.

The practical coatings on tablets were also carried out and disintegration time of the coated tablets, absorption of moisture and aging deterioration of the active ingredients were subsequently investigated. Most of the compounds have shown outstanding properties as protective-coating agents.

In the preceding reports, observations were made on the applicability of polyvinylpyridine derivatives and other compounds to protective-coating agents (1). All preparations coated with these compounds were easily dissolved in acid of gastric juice to release medicament. However, it is believed that there are marked differences in gastric acid secretion among individuals, particularly those patients with hypoacidity or anacidity. In this sense, it is feared that preparations coated with one of these compounds may be not disintegrated by gastric juice. In order to overcome this disadvantageous property of these compounds, preparations should be coated with such protective-coating agents as to be insoluble near the neutral pH but disintegrate favorably both in gastric juice of lower acidity and in intestinal juice.

Attempts to elucidate the properties of the polyampholytes obtained by the reaction of vinylpyridine or its alkyl derivatives with methacrylic or acrylic acid were made to examine their eligibilities for protective-coating agents.

Copolymerization of 2-vinylpyridine and methacrylic acid and physico chemical properties of the products have been reported by Alfrey (2) and Katchalsky (3), respectively. There have been other reports on the copolymerization of the main body of acrylonitrile with vinylpyridine, methacrylic acid or other vinyl compounds for textile-improving (4–6). On the other hand, no reported study has dealt with these products as coating agents for medicaments.

As starting monomers 4-vinylpyridine (4VP), 2-vinylpyridine (2VP), 2-methyl-5-vinylpyridine (MVP), and 2-vinyl-5-ethylpyridine (VEP) were used for basic components, whereas methacrylic acid (MAA) and acrylic acid (AA) were used for acidic components. Styrene (St), methylacrylate (MA), and acrylonitrile (AN) were used as other monomers.

Copolymers were synthesized from any two or three components using either a catalyst of benzoylperoxide (BPO) or α,α'-azo-bis-isobutyr-

Received December 15, 1961, from Osaka Research Laboratory, Tanabe Seiyaku Co. Ltd., Osaka, Japan.
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onitrile (AIBN) and their applicability to the protective-coating agents were studied.

EXPERIMENTAL

Materials.—Each monomer was rectified just before using. BPO and AIBN of pure grade in commerce were used without purification.

Synthesis of Copolymers.—A mixture of two or three monomers was dissolved in methanol and then polymerized at 50–60° and 60–70° in the presence of BPO and AIBN as catalyst, respectively, for 5 hr. To purify, each polymer thus obtained was dissolved in an adequate solvent, *e.g.*, methanol or acetone, and subsequently separated by pouring the solution into a large quantity of water. After repeating this reprecipitation, the polymer was dissolved in diluted hydrochloric acid and finally precipitated by adjusting the pH to isoelectric point with caustic alkali.

The copolymers with acrylic acid were synthesized by the presynthesis of the copolymers with MA followed by hydrolysis with an equimolecular amount of potassium hydroxide in acetone-methanol mixture for 5 hr. with heating.

Tables I, II, III, IV, and V show the yields of the copolymerizations and the analytical data of the copolymers.

Composition of Copolymers.—Vinylpyridine content in each copolymer was determined from nitrogen content (micro-Dumas method) and ultraviolet absorption (7, 8). Carboxyl content was determined by titration of copolymers with 0.1 *N* sodium methoxide solution in absolute pyridine using thymol blue as indicator (9).

TABLE I.—COPOLYMERIZATION OF 2-VINYL-5-ETHYL PYRIDINE

Monomer Ratio			Yield, %	Polymer Composition	
VEP Mol. %	Comonomer,	Mol. %		VEP Content, %	Acid Content, %
30	MAA	70	50.0	54.8	45.6
40		60	52.4	62.0	39.3
50		50	50.0	69.3	31.7
60		40	35.1	75.9	21.5
20	AA	80	47.0	45.1	54.1
40		60	70.7	49.6	45.8
60		40	49.2	69.5	36.2

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Monomer Ratio				Yield, %	Polymer Composition	
VEP Mol. %	MAA Mol. %	Comonomer,	Mol. %		VEP Content, %	Acid Content, %
20.0	50.0	St	30	35	56.1	29.3
30.0	40.0		30	61	54.8	19.9
35.0	35.0		30	75	46.1	17.1
40.0	30.0		30	68	37.3	11.8
50.0	20.0		30	57	76.0	8.6
60.0	10.0		30	53	75.5	3.1
28.6	21.4		50	55	37.0	20.0
34.3	25.7		40	56	56.1	11.2
45.7	31.3		20	55	63.5	19.5
51.4	38.6		10	58	65.5	16.1
28.6	21.4	AN	50	64	77.0	13.8
34.3	25.7		40	65	51.0	18.4
40.0	30.0		30	65	52.5	17.0
45.7	31.3		20	61	76.5	19.6
51.4	38.6		10	...	...	...
11.4	8.6	MA	80	70	16.6	47.4
17.2	12.8		70	54	20.4	43.0
22.9	17.1		60	56	41.5	14.0
28.6	21.4		50	61	66.7	13.7
34.3	25.7		40	60	64.6	16.5
40.0	30.0		30	47	72.0	18.0

TABLE III.—COPOLYMERIZATION OF 2-METHYL-5-VINYL PYRIDINE

Monomer Ratio				Yield, %	Polymer Composition	
MVP Mol. %	MAA Mol. %	Comonomer,	Mol. %		MVP Content, %	Acid Content, %
40.0	60.0	...		90.0	51.4	48.5
50.0	50.0	...		84.4	62.3	37.0
60.0	40.0	...		82.8	77.2	21.9
40.0	30.0	St	30	50.0	48.6	16.0
30.0	40.0		30	68.3	32.0	27.2
40.0	30.0	MA	30	72.0	42.0	15.35
28.6	21.4		50	65.3	55.2	18.96
22.6	17.1		60	72.4	59.8	17.10
17.2	12.8		70	50.0	62.4	16.00
40.0	30.0	AN	30	44.0	72.5	21.3
34.3	25.7		40	47.1	53.2	17.8
28.6	21.4		50	47.5	50.5	18.0
22.8	17.1		60	34.2	62.7	14.5
70.0	AA 30	St	30	37.7	68.8	23.4

TABLE IV.—COPOLYMERIZATION OF 2-VINYL PYRIDINE

Monomer Ratio				Yield, %	Polymer Composition	
2VP Mol. %	MAA Mol. %	Comonomer, Mol. %			2VP Content, %	Acid Content %
45.7	31.3	St	20	30.2	57.0	14.19
40.0	40.0		20	93.2	27.7	23.8
50.0	20.0		30	80.0	59.4	9.3
35.0	35.0		30	76.5	36.7	21.9
20.0	20.0		60	61.4	20.7	15.1
35.0	35.0	AN	30	28.3	45.5	22.0
34.7	25.7		40	20.3	81.3	15.7
34.3	25.7	MA	40	22.4	58.7	21.1
30.0	30.0		40	64.5	35.4	26.1
25.0	25.0		50	55.2	36.4	40.2
20.0	20.0		60	61.4	17.5	23.8
15.0	15.0		70	59.2	17.0	19.7
20.0	AA 20	St	60	63.8	21.4	13.9

TABLE V.—COPOLYMERIZATION OF 4-VINYL PYRIDINE

Monomer Ratio				Yield, %	Polymer Composition	
4VP Mol. %	MAA Mol. %	Comonomer, Mol. %			4VP Content, %	Acid Content, %
40.0	40.0	St	20	94.8	40.4	32.6
20.0	20.0		60	47.0	20.7	21.7
35.0	35.0	AN	30	86.5	45.5	26.8
40.0	30.0		30	21.4	54.5	23.40
34.3	25.7		40	45.0	91.0	10.28
15.0	15.0		70	33.3	17.0	45.6
40.0	30.0	MA	30	23.4	52.0	20.6
34.3	25.7		40	36.6	63.5	18.3
30.0	30.0		40	64.5	49.6	25.6
28.6	21.4		50	21.2	56.0	17.3
25.0	25.0		50	38.6	28.6	27.6
20.0	20.0		60	61.5	23.4	22.0
15.0	15.0		70	33.3	17.0	45.6

Isoelectric Zone.—A 100-mg. quantity of each copolymer was dissolved in 0.1% hydrochloric acid to make 100 ml. of the solution. A 50-ml. part of this solution was titrated with 0.1 *N* sodium hydroxide by using glass electrode pH meter (2). Isoelectric zone determined the pH value at which the solution began to get turbid and the pH value at which it became clear again.

Dynamic Viscoelasticity of Films.—Dynamic viscoelasticity of films was determined by "vibrating reed" method in a room at 20° and 60% R.H. The method of preparing the film of copolymers has been reported in a previous paper (10).

Water Vapor Permeability of Films.—The quantity of water permeated through the films per 24 hr. was examined by "cup method" specified in ASTM E-96-53T in a room at 25° and 92% R.H. (10).

Disintegration Test.—By the dipping method, the protective coating was applied on the starch-lactose tablets which were compressed in accordance with the method of a previous paper (10). Disintegration times of the coated tablets in distilled water, artificial gastric juice, artificial intestinal juice, and buffer solution from pH 2 to 9 were separately determined by the method specified in U.S.P. XVI. Acetic acid-sodium acetate system was used as the buffer solutions of pH 4 and 5, while Clark-Lubs system was employed as the buffer solutions with a pH other than pH 4 and 5.

The thickness of the coating film was calculated from the difference between the weight of coated and uncoated tablets. The composition of copolymers, solvents used in the coating process, and thickness of the films are shown in Table VI.

Aging Test.—Formulas of tablets used in the experiment for aging test were: (*a*) Carnitine chloride tablet containing 200 mg. of carnitine chloride was 9.6 mm. in diameter, 2.8 mm. of thickness and 260 mg. of total weight. (*b*) Acetylsalicylic acid tablet containing 115 mg. of acetylsalicylic acid had 8.0 mm. in diameter, 2.5 mm. of thickness and 150 mg. of total weight.

The dipping method was used to apply the protective coating to both tablets. Four copolymers selected from each series of VEP, MVP, 2VP, and 4VP were used as protective coating agents.

Coated carnitine chloride tablets were stored in a room at 25° and 90% R.H.; absorption of moisture was observed by measuring the change of tablet weight. Coated acetylsalicylic acid tablets were stored in a room at 37° and 85% R.H.; the amount of salicylic acid produced as a result of decomposition of acetylsalicylic acid was determined by means of its ultraviolet absorption (11).

Toxicity Test.—Each copolymer, suspended in water plus a small amount of sodium carboxymethylcellulose, was given to dd-strain male mice weighing 20 ± 1 Gm. by stomach tube. The mortality of mice 72 hr. after administration was observed to evaluate the toxicity of copolymers. The dose administered to the mice was the maximum which could be given.

RESULTS AND DISCUSSION

Solubility.—As shown in Table VII, two-component copolymers of vinylpyridine or its derivatives with MAA or AA were insoluble in water, soluble in

TABLE VI.—DATA FOR DISINTEGRATION TEST OF COATING

Tablet	Coating Agent: Basic Component, Mol. %		Acidic Component, Mol. %		Other Component, Mol. %		Coating Soln.[a] Concn., w/v %	Thickness of Film, μ
	VEP	40	MAA	30	St	30	10	108
		34		26	AN	40	10	108
		17		13	MA	70	5	150
		29		21		50	5	88
Starch-lactose	MVP	40	MAA	30	St	30	10	121
		40		30	AN	30	10	170
		17		13	MA	70	5	143
		23		17		60	5	111
	4VP	20	MAA	20	MA	60	10	308
	2VP	20	MAA	20	MA	60	10	230
	VEP	11	MAA	9	MA	80	10	41
Carnitine chloride	MVP	23	MAA	17	MA	60	10	31
	4VP	20	MAA	20	MA	60	10	48
	2VP	20	MAA	20	MA	60	10	42
Acetylsalicylic acid	VEP	11	MAA	9	MA	80	10	120
	MVP	23	MAA	17	MA	60	10	105

[a] Solvent utilized was chloroform and methyl alcohol (1:1).

TABLE VII.—SOLUBILITY OF COPOLYMER

Coating Agent: Vinyl Pyridine, Mol. %		Other Component, Mol. %		Solvent[a]: Methyl Alcohol	Ethyl Alcohol	Acetone	Benzene	Carbon Tetrachloride	Chloroform	Ethyl Acetate	Ether	Petroleum Ether	Artificial Gastric Juice	Artificial Intestinal Juice	Distilled Water	Isoelectric Zone
VEP	30	MAA	70	SS	I	I	I	I	I	I	I	I	S	S	I	3.8–7.2
	40		60	S	S	I	I	I	SS	I	I	I	S	S	I	4.1–7.6
	60		40	S	S	I	I	I	S	I	I	I	S	I	I	4.1–8.7
VEP	20	AA	80	SS	SS	I	I	I	I	I	I	I	S	S	I	3.8–7.2
	40		60	SS	SS	I	I	I	I	I	I	I	S	S	I	4.0–6.3
	60		40	SS	I	I	I	I	I	I	I	I	S	S	I	3.5–5.6
MVP	50	MAA	50	I	I	I	I	I	I	I	I	I	S	S	I	4.5–6.1
	60		40	SS	SS	I	G	SS	SS	SS	I	I	S	S	I	4.5–7.3
MVP	60	AA	40	S	S	SS	G	G	G	G	I	I	S	S	I	4.0–6.3
	70		30	S	S	SS	G	G	G	G	I	I	S	I	SS	4.5–9.0
2VP	60	MAA	40	G	G	SS	SS	G	G	I	I	I	S	I	SS	4.2–10.0
4VP	60	MAA	40	S	SS	I	I	S	I	I	I	I	S	S	I	3.9–6.5

[a] I = insoluble, S = soluble, SS = slightly soluble, and G = gelatinized or swollen.

weak acid and alkali, but very sparingly soluble in organic solvents, with a few exceptions, so that most of the compounds seemed to be unsuitable for protective coating agents.

On the other hand, three-component copolymers with St, AN or MA, as shown in Tables VIII, IX and X, were easily dissolved in organic solvents to form transparent films, were soluble in weak acid and alkali, but insoluble in near neutral. As seen in Table VIII, the copolymers composed of St in 30 mol. % content showed the highest solubility when the content ratio of VEP to MAA was 4:3 or 1:1. Therefore, various ratios of components for the synthesis of copolymers were later set up by maintaining the ratio of VEP to MAA; either 4:3 or 1:1.

Table XI represents the composition for synthesizing copolymers providing the most favorable solubility.

The copolymer films were strengthened with an increase of MA, St, or AN. Since copolymers containing up to 70 mol. % MA content were soluble, as indicated in Table XI, the films containing MA were considered the strongest.

As appended in Tables VIII, IX and X, each copolymer showed various ranges of the isoelectric zone according to the variation of composition. Therefore, a suitable copolymer could be selected freely depending on the property of medicament to be coated.

Water Vapor Permeability and Viscoelasticity of Films.—As indicated in Table XII, water vapor permeability of the film formed by these protective-coating agents was close to 10^{-4}–10^{-5} Gm. cm./cm.^{2}/24 hr. which were obtained by the protective-coating agents reported in a previous paper (10).

Less water vapor permeability of the films produced by three-component copolymers was noted as

compared with those produced by two-component copolymers in a same series of vinylpyridine derivatives. Furthermore, the copolymers with MA, St, or AN showed a decrease of water vapor permeability of the films with increasing the content of these components. Especially, the copolymers composed of MVP-MAA-MA and MVP-MAA-St showed the lowest water vapor permeability. It is believed that the lower water vapor permeability of films is desirable for a protective-coating agent.

Dynamic elasticity and dynamic viscosity of the films of copolymers, as indicated in Table XII, were approximately 10^{10} dynes/cm.^{2} and 10^6 poises, which are generally suitable for a protective-coating agent.

Disintegration of Protective Coated Tablets.—Disintegration tests of the tablets coated with a protective-coating agent were carried out in artificial gastric juice, intestinal juice, and also in the buffer solution to evaluate disintegration time and water resistance. The results of the disintegration tests are shown in Table XIII. All of the coated tablets showed a resistance to disintegration in distilled water for more than 3 hr. but disintegrated within 15 min. in artificial gastric juice and within 30 min. in artificial intestinal juice. The results of

TABLE VIII.—SOLUBILITY OF COPOLYMER

Coating Agent				Solvent[a]												
VEP, Mol. %	MAA, Mol. %	Other Comonomer,	Mol. %	Methyl Alcohol	Ethyl Alcohol	Acetone	Benzene	Carbon Tetrachloride	Chloroform	Ethyl Acetate	Ether	Petroleum Ether	Artificial Gastric Juice	Artificial Intestinal Juice	Distilled Water	Isoelectric Zone
20.0	50.0	St	30	G	I	I	I	I	G	I	I	I	I	I	I	...
30.0	40.0		30	S	S	I	SS	SS	S	S	I	I	S	I	I	4.4–9.2
35.0	35.0		30	S	S	I	SS	SS	S	S	I	I	S	S	I	4.3–7.2
40.0	30.0		30	S	S	SS	S	S	S	S	I	I	S	S	I	4.3–7.6
50.0	20.0		30	S	S	SS	S	S	S	S	I	I	S	G	G	4.3–8.2
60.0	10.0		30	S	S	I	I	I	S	I	I	I	S	G	G	4.3–8.1
28.6	21.4		50	G	G	I	G	I	G	I	I	I	S	I	I	4.4–9.8
34.3	25.7		40	S	S	SS	S	SS	S	S	SS	I	S	G	I	4.4–8.6
45.7	34.3		20	S	S	SS	S	SS	S	S	SS	I	S	G	I	4.6–8.7
51.4	38.6		10	S	S	I	S	I	S	S	I	I	S	I	I	4.3–10.0
28.6	21.4	AN	50	S	SS	SS	SS	I	S	S	I	I	S	I	I	4.9–9.0
34.3	25.7		40	S	S	SS	SS	I	S	S	I	I	S	SS	I	4.0–7.9
40.0	30.0		30	S	S	SS	SS	I	S	S	I	I	S	S	I	4.0–7.4
45.7	31.3		20	S	S	SS	SS	I	S	S	I	I	S	SS	I	4.3–8.0
51.4	38.6		10	S	S	SS	G	G	S	S	I	I	S	G	I	4.5–8.4
11.4	8.6	MA	80	S	S	S	SS	SS	S	S	SS	I	G	I	I	...
17.2	12.8		70	S	S	SS	SS	SS	SS	S	SS	I	S	S	I	3.7–6.0
22.9	17.1		60	S	S	SS	SS	SS	SS	S	SS	I	S	S	I	4.0–7.2
28.6	21.4		50	S	S	SS	SS	SS	S	S	SS	I	S	SS	I	3.9–7.9
34.3	25.7		40	S	S	SS	SS	I	S	S	SS	I	S	S	I	4.2–7.5
40.0	30.0		30	S	S	SS	SS	I	S	S	SS	I	S	G	I	4.7–8.3

[a] I = insoluble, S = soluble, SS = slightly soluble, and G = gelatinized or swollen.

TABLE IX.—SOLUBILITY OF COPOLYMER

Coating Agent				Solvent[a]												
MVP, Mol. %	MAA, Mol. %	Other Comonomer,	Mol. %	Methyl Alcohol	Ethyl Alcohol	Acetone	Benzene	Carbon Tetrachloride	Chloroform	Ethyl Acetate	Ether	Petroleum Ether	Artificial Gastric Juice	Artificial Intestinal Juice	Distilled Water	Isoelectric Zone
40	30	St	30	S	I	I	G	G	I	I	I	I	S	S	I	3.5–6.1
17.2	12.8	MA	70	S	I	I	S	G	I	I	I	I	S	S	I	3.5–6.1
22.8	17.1		60	S	I	I	G	I	I	I	I	I	S	S	I	4.3–6.4
28.6	21.4		50	S	I	I	G	I	I	I	I	I	S	S	I	4.5–6.4
34.3	25.7		40	S	I	I	G	I	I	I	I	I	S	S	I	4.3–6.3
40.0	30.0		30	I	I	I	G	I	I	I	I	I	S	S	I	3.8–5.8
22.8	17.1	AN	60	S	I	I	G	I	I	I	I	I	SS	S	I	...
34.3	25.7		40	G	I	I	G	I	I	I	I	I	S	S	I	4.3–6.3
40.0	30		30	I	I	I	G	I	I	I	I	I	S	S	I	4.4–6.4

I = insoluble, S = soluble, SS = slightly soluble, and G = gelatinized or swollen.

TABLE X.—SOLUBILITY OF COPOLYMER

Coating Agent					Solvent[a]											
4VP or 2VP, Mol. %		MAA, Mol. %	Other Comonomer, Mol. %		Methyl Alcohol	Ethyl Alcohol	Acetone	Benzene	Chloroform	Dioxane	Ether	Petroleum Ether	Artificial Gastric Juice	Artificial Intestinal Juice	Distilled Water	Isoelectric Zone
4VP	20	20	St	60	G	G	SS	G	S	S	I	I	S	SS	I	3.3–8.0
	40	40		20	SS	I	I	G	G	I	I	I	S	S	I	3.6–6.7
	35	35	AN	30	S	SS	I	G	G	I	I	I	S	S	I	3.8–6.1
	15	15	MA	70	S	SS	G	I	G	G	I	I	S	S	I	3.2–6.7
	25	25		50	S	S	I	S	G	I	I	I	S	S	I	3.5–6.4
	40	30		30	S	SS	I	G	G	I	I	I	S	S	I	3.9–6.5
2VP	20	20	St	60	S	SS	G	G	G	S	I	I	I	I	I	...
	35	35		30	S	SS	I	I	G	SS	I	I	S	SS	I	3.6–8.2
	35	35	AN	30	S	SS	I	I	G	SS	I	I	S	S	I	3.9–6.2
	15	15	MA	70	S	SS	G	I	G	G	I	I	S	S	I	3.5–6.5
	25	25		50	S	S	I	S	G	I	I	I	S	S	I	3.5–6.5
	30	30		40	S	SS	I	I	G	SS	I	I	S	S	I	3.6–6.4

[a] I = insoluble, S = soluble, SS = slightly soluble, and G = gelatinized or swollen.

TABLE XI.—BEST COMPOSITION OF COPOLYMER FOR SOLUBILITY

Mol. ratio of VP:MAA	Mol. % of other component	
VEP-MAA (4:3)	St	30
	AN	30
	MA	40–70
MVP-MAA (4:3)	St	30
	AN	30–40
	MA	40–70
2VP-MAA (1:1)	St	30
	AN	30
	MA	40–70
4VP-MAA (1:1)	St	20
	AN	30
	MA	30–70

disintegration tests in the buffer solutions showed generally same tendency within the pH range of the isoelectric zone determined previously.

Based on the foregoing, these copolymers seemed to be suitable protective-coating agents having an isoelectric zone around neutral and enough water resisting property suited for the purpose of this study. The tablets coated with these copolymers might be expected to disintegrate in a hypoacidic stomach or in the intestine even if it passed through the stomach without disintegration and subsequently exert therapeutic effect. This was noted in oral administration tests using vitamin B_2 tablets coated with these copolymers. These data will be disclosed in the near future.

Protective Effect on Active Ingredients.—Experiments to assess a protective effect of the copolymers against absorption of moisture were carried out by coating the carnitine chloride tablets, which are remarkably hygroscopic. Fig. 1 sets forth the results.

Hygroscopicity of the coated tablets was greatly reduced as compared with the uncoated control tablets. Both the appearance and hardness of the coated tablets remained unchanged while the uncoated tablets were softened, followed by change of form.

As described in Fig. 2, the results of aging test on the acetylsalicylic acid tablets coated with the copolymers revealed that the tablets were apparently stabilized when compared with stability of the uncoated tablets. The coated tablets disclosed a favorable result with reasonable disintegration time.

Toxicity.—The toxicity of polymers of vinylpyridine or vinylpyridine alkyl derivatives and copolymers of these monomers with VAc, AN, MA, or St has been reported in a previous paper (10). As summarized in Table XIV of this paper, no acute toxic

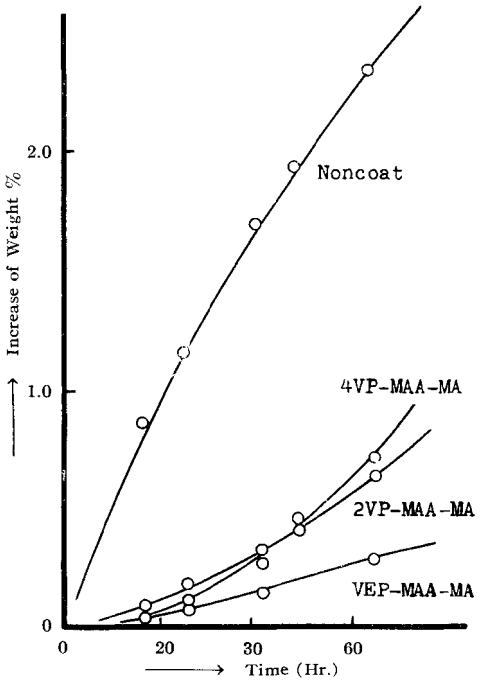


Fig. 1.—Hygroscopic curve of carnitine chloride tablet at 25°, 90% R.H.

TABLE XII.—WATER VAPOR PERMEABILITY AND VISCOELASTICITY OF FILMS

Composition of Coating Agent, Mol. %						Water Vapor Permeability, $\times 10^{-4}$ Gm. cm./cm.^{2}/24 hr.	Dynamic Modulus, $\times 10^{10}$ dyne/cm.^{2}	Dynamic Viscosity, $\times 10^6$ poise
VEP	17	MAA	13	MA	70	1.1	1.0	1.6
	25		13		60	1.0	1.0	6.5
	29		21		50	2.2	1.3	2.4
MVP	60	MAA	40			1.5	1.7	1.8
	40		30	St	30	0.1	1.2	0.6
	40		30	AN	30	0.9	1.8	5.6
	23		17	MA	60	0.3	2.3	1.1
	29		21		50	0.6	2.5	1.5
	34		26		40	0.2	5.5	1.5
4VP	20	MAA	20	MA	60	0.3	1.5	1.4
2VP	20	MAA	20	MA	60	0.3	1.2	1.3

TABLE XIII.—DISINTEGRATION TEST

Composition of Coating Agent, Mol. %						Disintegration Time		
						Distilled Water, hr.	Artificial Gastric Juice, min.	Artificial Intestinal Juice, min.
VEP	40	MAA	30	St	30	3	13	30
	34		26	AN	40	3	9	30
	17		13	MA	70	3	12	22
	29		21		50	3	7	30
MVP	40	MAA	30	St	30	3	9	30
	40		30	AN	30	3	13	30
	17		13	MA	70	3	13	22
	23		17		60	3	15	28
	29		21		50	3	10	28
	34		26		40	3	14	30
4VP	20	MAA	20	MA	60	3	12	28
2VP	20	MAA	20	MA	60	3	10	30

action upon mice was observed after the oral administration of four kinds of the typical copolymers with MAA and MA.

SUMMARY AND CONCLUSIONS

1. The polyampholites of vinylpyridine or its alkyl derivatives with acrylic or methacrylic acid and the copolymers of these compounds with other vinyl derivatives were synthesized to obtain new protective-coating agents.

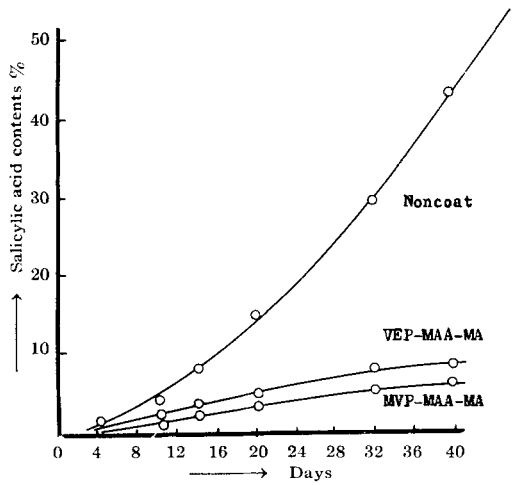


Fig. 2.—Aging of acetylsalicylic acid in acetylsalicylic acid tablets at 37°, 85% R.H.

TABLE XIV.—ACUTE TOXICITY TEST IN MICE[a]

No. of Animals	Protective-Coating Agent	Dose, mg./Kg.
5	VEP-MAA-MA	2000
5	MVP-MAA-MA	2000
5	2VP-MAA-MA	2000
5	4VP-MAA-MA	2000

[a] No deaths 72 hr. after administration.

2. These copolymers were dissolved in organic solvents to form transparent films which were soluble in both acidic and alkaline solutions but insoluble in an approximately neutral solution. Water vapor permeability through the copolymer films was found to be very low.

3. It was found that the copolymers synthesized had adequate viscoelasticity to serve as protective-coating agents.

4. Sufficient protection against absorption of moisture and aging deterioration of the active ingredients was provided by the copolymer coatings.

5. The tablet coatings were insoluble only in an approximately neutral solution, but seemed to be disintegrated in stomach with hypoacidity and in the intestine to produce the desired therapeutic effect.

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Tablets of Pyrilamine Resin Adsorbate with Aspirin and Vitamin C

By SHELDON SIEGEL, ROY H. REINER, JAMES A. ZELINSKIE, and EDWARD J. HANUS

Marked changes in physical appearance manifested by dry formulations containing aspirin, an antihistamine, and vitamin C during an accelerated temperature stability study are discussed. The stabilization afforded by a resin adsorbate of a commonly used antihistamine, pyrilamine, is noted. Discrepancies in the results obtained from stability studies of these preparations have been traced to the efficiency of the closure utilized in the packaging of the product, as well as the methods employed in the pretreatment, sampling, and evaluation of test samples. The implications of these observations from a developmental standpoint are discussed.

THE AVAILABILITY of ion exchange resins suitable for oral administration has presented the formulator with a means of tailoring products in a way not heretofore possible. These materials are currently available in a wide range of types and mesh specifications and present a new means of complexation of an active ingredient which can lead to a significant enhancement in both the stability of the medicament and in the manipulation of its release pattern. It is with a variation of the former case, the reduction of an undesirable incompatibility of salts of pyrilamine in common formulations, that this paper concerns itself.

Tablet and capsule formulations for the symptomatic treatment of the common cold constitute a significant segment of the ethical and proprietary drug market. These products usually contain aspirin, an antihistamine, and vitamin C. The development of such products is complicated by interactions between the ingredients, resulting in marked changes in the physical appearance of the preparation after short storage intervals. Certain antihistamines have been shown to be incompatible in dry dosage forms containing aspirin and/or vitamin C (1, 2). This interaction is manifested by softening and darkening of tablets, surface crystal growth, and an acetous odor. Methods such as compression coating, multilayering, and pan coating, which overcome the existing problems by physical separation of the reactants, have been utilized to produce products having an acceptable shelf life. We have investigated resin adsorbates of pyrilamine for possible reduction in the degree of incompatibility in formulations of this type.

Received March 26, 1962, from Merck & Co., Inc., Chemical Division, Product Development Laboratories, Rahway, N. J.
Accepted for publication May 4, 1962.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
Grateful appreciation is given to Miss B. Feller and Mr. J. Kanora for the chemical assays, and to Dr. G. Litt and Mr. J. Blodinger for their helpful suggestions.

EXPERIMENTAL

Analytical Methods

Pyrilamine Resin Adsorbate.—Accurately weigh a sample of finely ground tablets equivalent to 25 mg. of pyrilamine maleate. Disperse in 25 ml. of 2 *N* hydrochloric acid and shake vigorously for 30 minutes at room temperature. Extract with three 15-ml. portions of chloroform, discarding the chloroform layers. Add 5 ml. of 50% potassium hydroxide to the acid layer, shake well, and let stand at room temperature for 15 minutes. Extract with three 15-ml. portions of chloroform. Extract the combined chloroform extracts twice with 25 ml. of 1 *N* hydrochloric acid and measure the absorbance at 315 mμ in a suitable spectrophotometer. Use 1 *N* hydrochloric acid as the reference solution.

$$\frac{A \text{ of sample at } 315 \text{ m}\mu \times 10}{0.205 \times \text{No. of tablets}} = \text{mg. per tablet as pyrilamine maleate}$$

Ascorbic acid.—Ascorbic acid was assayed by the official U.S.P. XVI method.

Acetylsalicylic and Salicylic Acids.—A rapid spectrophotometric assay of acetylsalicylic and salicylic acids was utilized in this investigation (3). It is to be noted that the utilization of this assay procedure in preparations containing pyrilamine is somewhat inaccurate because of the interference exhibited by the absorbance of pyrilamine at the 308 mμ peak. To simplify the numerous assays involved, this interference was considered negligible; however, the values obtained should not be considered absolute.

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Marked changes in physical appearance manifested by dry formulations containing aspirin, an antihistamine, and vitamin C during an accelerated temperature stability study are discussed. The stabilization afforded by a resin adsorbate of a commonly used antihistamine, pyrilamine, is noted. Discrepancies in the results obtained from stability studies of these preparations have been traced to the efficiency of the closure utilized in the packaging of the product, as well as the methods employed in the pretreatment, sampling, and evaluation of test samples. The implications of these observations from a developmental standpoint are discussed.

THE AVAILABILITY of ion exchange resins suitable for oral administration has presented the formulator with a means of tailoring products in a way not heretofore possible. These materials are currently available in a wide range of types and mesh specifications and present a new means of complexation of an active ingredient which can lead to a significant enhancement in both the stability of the medicament and in the manipulation of its release pattern. It is with a variation of the former case, the reduction of an undesirable incompatibility of salts of pyrilamine in common formulations, that this paper concerns itself.

Tablet and capsule formulations for the symptomatic treatment of the common cold constitute a significant segment of the ethical and proprietary drug market. These products usually contain aspirin, an antihistamine, and vitamin C. The development of such products is complicated by interactions between the ingredients, resulting in marked changes in the physical appearance of the preparation after short storage intervals. Certain antihistamines have been shown to be incompatible in dry dosage forms containing aspirin and/or vitamin C (1, 2). This interaction is manifested by softening and darkening of tablets, surface crystal growth, and an acetous odor. Methods such as compression coating, multilayering, and pan coating, which overcome the existing problems by physical separation of the reactants, have been utilized to produce products having an acceptable shelf life. We have investigated resin adsorbates of pyrilamine for possible reduction in the degree of incompatibility in formulations of this type.

Received March 26, 1962, from Merck & Co., Inc., Chemical Division, Product Development Laboratories, Rahway, N. J.
Accepted for publication May 4, 1962.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
Grateful appreciation is given to Miss B. Feller and Mr. J. Kanora for the chemical assays, and to Dr. G. Litt and Mr. J. Blodinger for their helpful suggestions.

EXPERIMENTAL

Analytical Methods

Pyrilamine Resin Adsorbate.—Accurately weigh a sample of finely ground tablets equivalent to 25 mg. of pyrilamine maleate. Disperse in 25 ml. of 2 *N* hydrochloric acid and shake vigorously for 30 minutes at room temperature. Extract with three 15-ml. portions of chloroform, discarding the chloroform layers. Add 5 ml. of 50% potassium hydroxide to the acid layer, shake well, and let stand at room temperature for 15 minutes. Extract with three 15-ml. portions of chloroform. Extract the combined chloroform extracts twice with 25 ml. of 1 *N* hydrochloric acid and measure the absorbance at 315 mμ in a suitable spectrophotometer. Use 1 *N* hydrochloric acid as the reference solution.

$$\frac{A \text{ of sample at } 315 \text{ m}\mu \times 10}{0.205 \times \text{No. of tablets}} = \text{mg. per tablet as pyrilamine maleate}$$

Ascorbic acid.—Ascorbic acid was assayed by the official U.S.P. XVI method.

Acetylsalicylic and Salicylic Acids.—A rapid spectrophotometric assay of acetylsalicylic and salicylic acids was utilized in this investigation (3). It is to be noted that the utilization of this assay procedure in preparations containing pyrilamine is somewhat inaccurate because of the interference exhibited by the absorbance of pyrilamine at the 308 mμ peak. To simplify the numerous assays involved, this interference was considered negligible; however, the values obtained should not be considered absolute.

Sample Preparation and Treatment

Pyrilamine base was adsorbed on a weakly acidic carboxylic acid ion exchange resin (XE-64)[1] in varying concentrations. The pyrilamine resin adsorbate formed (PRA) is a free flowing, off-white, essentially odorless powder. Economic and formulation considerations resulted in the utilization of a resin product containing the equivalent of from 61.2 to 64.0% pyrilamine maleate.

Hard gelatin capsules of PRA, aspirin, and vitamin C; PRA, aspirin, and sodium ascorbate; and controls containing pyrilamine maleate in place of the resin adsorbate were prepared and placed in storage at several temperature stations. The ratio of the ingredients in the formulations placed on this study is summarized in Table I.

TABLE I.—RATIO OF INGREDIENTS IN FORMULATIONS IN STUDY

	Formulation			
	1	2	3	4
Pyrilamine resin adsorbate, mg.	20.4[a]	...	20.4[a]	...
Pyrilamine maleate, mg.	...	12.5	...	12.5
Aspirin, mg.	300	300	300	300
Ascorbic acid, mg.	...	...	50	50
Sodium ascorbate, mg.	56.24[b]	56.24[b]	...	...

[a] Equivalent to 12.5 mg. pyrilamine maleate. [b] Equivalent to 50 mg. ascorbic acid.

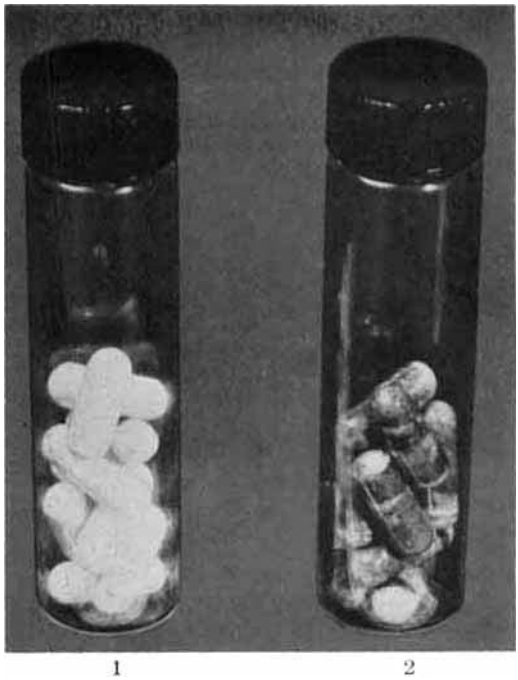
Compressed tablets containing aspirin powder, 300 mg., sodium ascorbate, 28 mg. (equivalent to 25 mg. ascorbic acid); PRA, 20.4 mg. (equivalent to 12.5 mg. pyrilamine maleate); and a control containing the maleate in place of the resin adsorbate were prepared by dry slugging methods. Corn starch, 10%; dried talcum, 5%; and Cab-O-Sil,[2] 0.5% were utilized as tableting aids. These were also placed in storage at elevated temperatures and observed periodically for gross physical changes.

As several apparent inconsistencies were disclosed in the interpretation of the observations of both the capsule and tablet formulations, a somewhat more detailed study was undertaken. The relationship of such factors as preheating of the tablets prior to packaging, the type of closure used on the container, and the frequency of inspection of the samples were investigated.

DISCUSSION

In all preparations studied under accelerated conditions, those containing the resin adsorbate of the antihistamine showed the least physical change. Figure 1 shows the difference in appearance between the capsule formulation containing PRA, aspirin, and sodium ascorbate, and that with pyrilamine maleate, aspirin, and sodium ascorbate after storage for 1 month at 45°. The enhancement of physical appearance by using the resin form is immediately apparent. It was also observed that preparations containing sodium ascorbate were far superior in this same respect to those containing ascorbic acid.

In the initial investigation, several inconsistent results were noted concerning the stability of the tablets which were not readily explainable. A significant lack of uniformity was observed between



1 2

Fig. 1.—1, Capsules of pyrilamine resin adsorbate, aspirin, and sodium ascorbate; 2, Capsules of pyrilamine maleate, aspirin, and sodium ascorbate, after 1 month of storage at 45°.

samples stored in bottles with polyethylene snap caps and those stored with Bakelite screw caps. Tablets in containers with the polyethylene closures were superior in physical appearance to those in containers sealed by screw caps. An investigation of the relationship of closure to stability was then undertaken and Fig. 2 illustrates the difference in physical stability experienced with the same formulation under different packaging conditions after storage for 2 months at 40°. Note the evidence of decomposition in the vial sealed with a nonporous cap (A), the improvement when a permeable polyethylene closure is used (C), and the absence of physical change when the containers are left open (E and F).

Although it is well known that polyethylene will allow the passage of air and other volatile material, the significance of this in common stability studies of this type has not, to our knowledge, been given sufficient recognition. Based on our accelerated studies with formulations of this type, it would then appear that the more permeable the container closure, the greater the physical stability of the contents. The following explanation is offered for this phenomenon. Under the temperature conditions imposed, this permeability would allow the escape of moisture from the formulations, thereby effectively removing the component responsible for the hydrolytic degradation of aspirin. In addition, the open container (E) and, to a lesser degree, the container with a permeable closure (polyethylene snap cap), permit the escape of the acidic gaseous degradation products resulting from aspirin hydrolysis. This would suggest that the autocatalytic degradation of aspirin is promoted by the presence of these acidic degradation products. It should also be men-

[1] Rohm and Haas Co., Philadelphia, Pa.
[2] Godfrey L. Cabot, Inc., Boston, Mass.

tioned that an acidic environment within the container would promote the desorption of the pyrilamine from the resin in the form of an acid salt, which was previously shown to be less desirable in preparations of this type.

Several other very practical considerations which may be explained on the above basis were observed during this investigation. Filled containers appeared to exhibit greater instability than partially filled ones (see Fig. 2). This indicates that this difference is due to the entrapment of significantly more volatile decomposition products, hence increasing the rate of degradation. Further corroboration of this postulate is taken from the observation that samples which had been opened frequently to check stability appeared more stable than counterparts which had remained unopened.

It was reasoned that if the foregoing explanation for the marked stability differences was valid, preheating the tablets prior to packaging should result in a significant enhancement of stability. Figure 3 illustrates the result of heating tablets for 20 and 40 hours at 50° before packaging and subsequent storage at 45° for 1 month. The unheated sample (G) underwent considerable physical degradation, evidenced by darkening of the tablets and appearance of a large number of crystals, shown by infrared spectroscopy to be salicylic acid. The heated samples (H and I) show no change in physical appearance. It thus appears that this preheating procedure resulted in the expulsion of both moisture and aspirin degradation products and, in this way, exerted a stabilizing influence on the formulation in addition to that achieved by the use of the resin adsorbate of pyrilamine. The chemical stability of this formulation is summarized in Table II. It is clear from the analytical data that the gross physical appearance of the preparation served as a valid indicator for the actual decomposition of the formulation.

It is felt that the observations reported here have strong implications from the developmental standpoint. The type of container closure and degree of fill are obviously of prime importance in evaluation of these products by accelerated temperature studies. It is interesting that formulations of this type appear to reverse the well documented belief that, to a large extent, stability is dependent upon the ability of the container to isolate the ingredients from the environment. It can be seen that a stability study that does not take into consideration factors of this type is quite likely to lead to fallacious extrapolations.

SUMMARY

Formulations containing pyrilamine resin adsorbate, aspirin, and vitamin C were found to be less subject to physical change in accelerated stability studies than control formulations containing the maleate salt of pyrilamine. An apparent lack of uniformity in the stability of such formulations has been related to the selection of container closures, container fill,

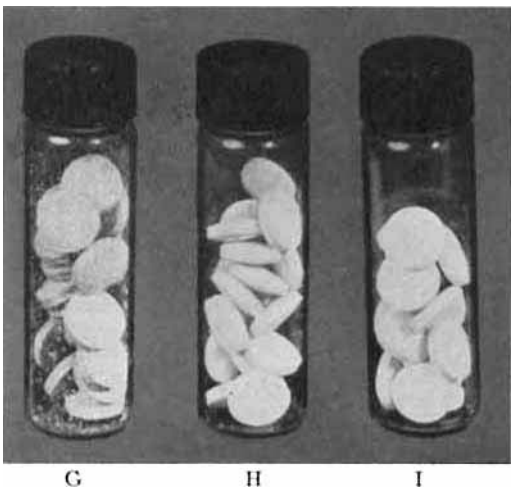


Fig. 3.—Effect of preheating on the stability of tablets containing pyrilamine resin adsorbate, aspirin, and sodium ascorbate after 1 month storage at 45°.

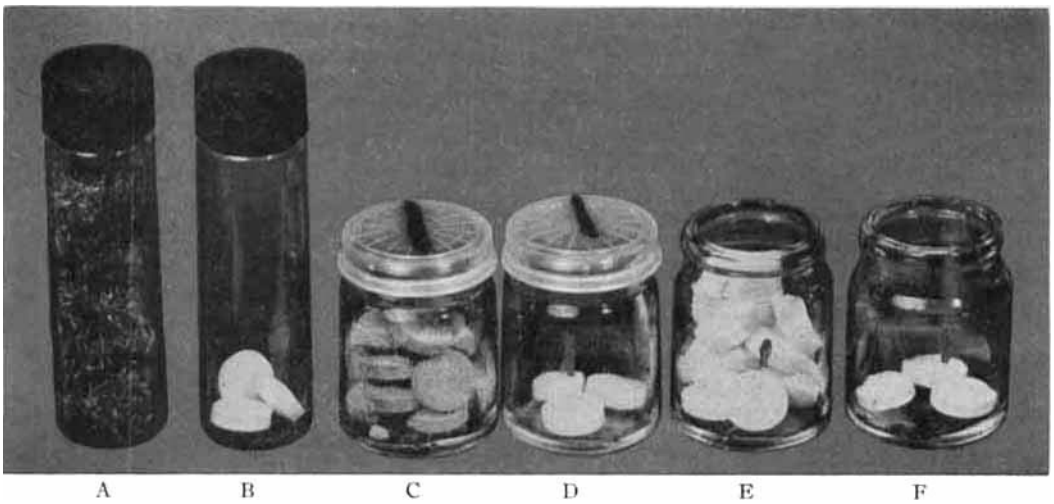


Fig. 2.—Comparative stability of a formulation containing pyrilamine resin adsorbate, aspirin, and sodium ascorbate under different packaging conditions after 2 months storage at 40°.

TABLE II.—CHEMICAL STABILITY OF FORMULATIONS[a]

Formula G	Initial Assay	60°C. 1 wk.	45°C. 1 wk.	45°C. 4 wk.	45°C. 8 wk.	45°C. 10 wk.	45°C. 12 wk.
Acetylsalicylic acid	319	271	314	297	273	267	252
Salicylic acid	7.0	76	10.5	18.7	58.0	77.0	82.0
Pyrilamine resin adsorbate (expressed as pyrilamine maleate)	12.7	...	12.7	...	11.8	...	11.0
Sodium ascorbate (expressed as ascorbic acid)	24.6	...	23.4	...	13.8	...	1.2
Formula H							
Acetylsalicylic acid	317	317	308	304	321	322	310
Salicylic acid	7.4	16	10.3	7.9	9.2	8.7	9.4
Pyrilamine resin adsorbate (expressed as pyrilamine maleate)	12.8	...	12.7	...	12.2	...	12.2
Sodium ascorbate (expressed as ascorbic acid)	25.6	...	23.8	...	24.8	...	24.7
Formula I							
Acetylsalicylic acid	324	298	316	305	317	314	314
Salicylic acid	7.6	16	9.6	8.7	7.6	8.6	8.8
Pyrilamine resin adsorbate (expressed as pyrilamine maleate)	12.7	...	12.6	...	12.7	...	12.4
Sodium ascorbate (expressed as ascorbic acid)	25.1	...	23.4	...	24.2	...	23.8

[a] Assay results reported as milligrams per tablet.

and sample handling techniques. The heat treatment of tablets prior to packaging has been shown to enhance further the stability of such products when stored at elevated temperatures.

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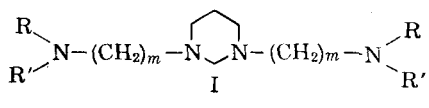
(1) Yamamoto, R., and Takahashi, T., *Ann. Repts. Shionogi Res. Lab.*, 4, 79(1954).
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Hexahydropyrimidines I. Preparation of 2-Substituted-1,3-bis(*p*-dimethylaminobenzyl)-hexahydropyrimidines

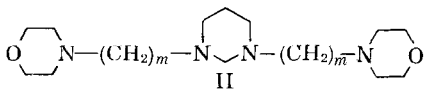
By JOHN H. BILLMAN and LINNEAUS C. DORMAN†

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ALTHOUGH the synthesis of hexahydropyrimidines from 1,3-diamines and carbonyl compounds has been described for some time by a number of workers (1–7), a review of the literature reveals that relatively few hexahydropyrimidines have been prepared and examined for medicinal activity. Two particularly interesting publications by Van Hook and Craig report that 1,3-bis(dialkylaminoalkyl)- (I) (8) and 1,3-bis(heterocyclicaminoalkyl)hexahydropyrimidines (II)(9) possess antifungal, antibacterial and



I



II

antiviral activity.

Owing to the relative ease with which these derivatives were prepared and their structural significance, it was of interest to determine if hexahydropyrimidines substituted in the 1-,2-, and 3-positions might also display similar, if not superior, pharmacological activity to the 1,3-di-

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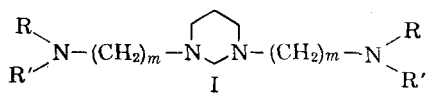
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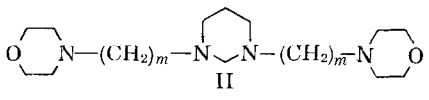
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I



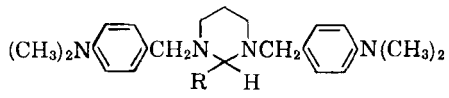
II

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Owing to the relative ease with which these derivatives were prepared and their structural significance, it was of interest to determine if hexahydropyrimidines substituted in the 1-,2-, and 3-positions might also display similar, if not superior, pharmacological activity to the 1,3-di-

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TABLE I.—2-SUBSTITUTED-1,3-BIS-(*p*-DIMETHYLAMINOBENZYL)HEXAHYDROPYRIMIDINES

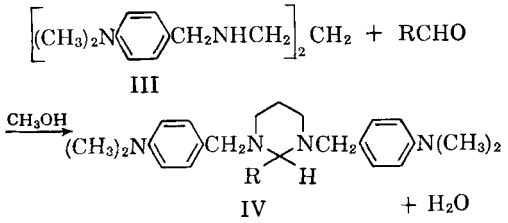


R	Yield, %	M.p., °C.	Formula	Nitrogen, % Calcd.	Found
3,4-Dimethoxyphenyl[a]	54	118–119	$C_{30}H_{40}N_4O_2$	11.47	11.67
p-Dimethylaminophenyl[a]	53	149.5–151	$C_{30}H_{41}N_5$	14.85	14.95
p-Nitrophenyl[a]	38	132–133	$C_{28}H_{35}N_5O_2$	14.79	14.85
3,4-Methylenedioxyphenyl[a]	60	152–153.2	$C_{29}H_{36}N_4O_2$	11.85	11.85
p-Methoxyphenyl[b]	73	125.2–126.2	$C_{29}H_{38}N_4O$	12.22	12.18
m-Hydroxyphenyl[b]	56	139–140.5 d.	$C_{28}H_{36}N_4O$	12.60	12.68
o-Hydroxyphenyl[b]	79	143–144	$C_{28}H_{36}N_4O$	12.60	12.66
3-Thienyl[b]	61	151.2–152	$C_{26}H_{34}N_4S$	12.87	12.98
2-Propyl[b]	70	107.3–108.3	$C_{25}H_{38}N_4$	14.10	14.48
p-Acetamidophenyl[b]	52	179.5–181	$C_{30}H_{39}N_5O$	14.42	14.20

[a] Procedure (*B*). [b] Procedure (*A*).

substituted hexahydropyrimidines, (I) and (II).

To this end, a series of 2-substituted-1,3-bis(*p*-dimethylaminobenzyl)hexahydropyrimidines (IV) (Table I) were prepared by allowing the desired aldehydes to react with 1,3-bis(*p*-dimethylaminobenzylamino)propane according to the following equation. The reason for selecting the hexahydropyrimidines we did is that the N,N-dimethylaminobenzyl grouping resembles in a way



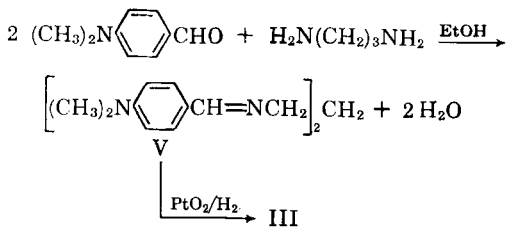
III

IV

the side-chains in the 1,3-bis(dialkylaminoalkyl)-hexahydropyrimidines (I) and 1,3-bis(heterocyclicaminoalkyl)hexahydropyrimidines (II). All three series of compounds have a tertiary-amino group at the end of the side-chains. In addition, the benzyl group should contribute to the solubility of the compounds to about the same extent as compounds of I and II with an average of five methylene groups in the side-chains.

Methanol was a convenient solvent for the ring closure reaction for producing the hexahydropyrimidines in yields of 33–78%. All of the compounds were solids and it was found that they could be hydrolyzed with dilute acid to regenerate the original reactants. In general the condensation reactions proceeded smoothly and rapidly at room temperature or on heating at moderate temperatures. However, difficulties were experienced with vanillin and furfural as these aldehydes failed to yield solid derivatives with 1,3-bis(*p*-dimethylaminobenzylamino)propane. Instead, dark viscous oils separated from their reaction mixtures and no pure products could be isolated.

The 1,3-bis(*p*-dimethylaminobenzylamino)propane (III) was prepared in two steps from trimethylenediamine and *p*-dimethylaminobenzaldehyde. In the initial step, the diamine was condensed with two moles of the aldehyde to produce the di-Schiff base, 1,3-bis(*p*-dimethylaminobenzylideneamino)propane (V). Reduction of the di-Schiff base with hydrogen over platinum oxide yielded the desired reagent.



V

III

The results to date indicate that none of the 2-substituted-1,3-bis(*p*-dimethylaminobenzyl)-hexahydropyrimidine derivatives possess antifungal or antibacterial activity. Other derivatives are now being prepared for testing.

EXPERIMENTAL[1]

Aldehydes.—Either reagent grades of aldehydes were used or they were purified by recrystallization from appropriate solvents. 3-Thiophenealdehyde was prepared by hydrolysis of its hexamethylenetetraammonium chloride salt (10) and used without further purification.

1,3 - Bis(*p*-dimethylaminobenzylideneamino)-

[1] Melting points were taken in open capillaries and are corrected. Microanalyses were performed by Miss J. Dickey of the Chemistry Department of Indiana University and/or by Midwest Microlab, Inc., Indianapolis, Ind.

propane (V).—To a refluxing solution of 64.3 Gm. (0.430 mole) of *p*-dimethylaminobenzaldehyde in 225 ml. of absolute ethanol was added a solution of 13.1 Gm. (0.177 mole) of trimethylenediamine in 30 ml. of absolute ethanol during a 30-min. period. The reaction mixture was refluxed an additional 30 minutes and the crystals which formed on cooling were collected and washed with ethanol; yield, 57.1 Gm. (96%), m.p. 144–145°. Recrystallization from absolute ethanol provided 45 Gm. (74%) of white crystals, m.p. 144.5° to 145°, $\lambda_{max.}^{KBr} = 6.05\mu$ (=C=N—).

Anal.—Calcd. for $C_{21}H_{28}N_4$: N, 16.65. Found: N, 16.59.

1,3 - Bis(*p*-dimethylaminobenzylamino)propane (III).—A mixture of 23.0 Gm. (0.069 mole) of 1,3 - bis(*p*-dimethylaminobenzylideneamino)propane, 1 Gm. of platinum oxide and 150 ml. of absolute ethanol was reduced at room temperature in a low pressure Parr hydrogenator with an initial hydrogen pressure of 47 p.s.i. Approximately 35 minutes were required to complete the reduction. The catalyst was removed by filtration and the filtrate was treated with 100 ml. of absolute ethanol, then slowly, while stirring vigorously, with 34 ml. of concentrated hydrochloric acid. The precipitated hydrochloride salt was removed by filtration, washed with absolute ethanol, dried partially under suction and completely in a vacuum desiccator over sodium hydroxide. The yield of crude 1,3-bis(*p*-dimethylaminobenzylamino)propane tetrahydrochloride monohydrate was 33.6 Gm. (98%). An analytical sample was obtained by two recrystallizations of the crude material from a mixture of 10% hydrochloric acid and absolute ethanol, 1:10, (the hot amine salt dilute hydrochloric acid solution was filtered into the absolute ethanol); decompn. point, 240–242° (brass Maquenne block).

Anal.—Calcd. for $C_{21}H_{38}Cl_4N_4O$: N, 11.11; Cl, 28.12. Found: N, 11.03; Cl, 28.10.

The free tetramine was recovered from its aqueous salt solution by precipitation with 10% sodium hydroxide. It was collected on a filter, washed with water, partially dried (rubber dam), then quickly transferred to a vacuum desiccator where drying was completed over sodium hydroxide. The tetramine occasionally softened in the desiccator, but solidified again on repeated drying *in vacuo*. The tetramine liquifies in air, but remains in solid form when kept in a desiccator over solid alkali. An analytical sample of 1,3-bis(*p*-dimethylaminobenzylamino)propane was prepared from its purified hydrochloride salt with aqueous alkali and dried *in vacuo* over sodium hydroxide and Ascarite, m.p. 38–40° (sealed capillary).

Anal.—Calcd. for $C_{21}H_{34}N_4$: C, 74.07; H, 9.47; N, 16.46. Found: C, 73.72; H, 9.57; N, 16.45.

2 - Substituted - 1,3 - bis(*p*-dimethylaminobenzyl)hexahydropyrimidines (IV).—The 2-(2-propyl)- derivative was prepared by the following procedure (*A*). To a stirred solution of 5.0 Gm. (0.015 mole) of 1,3-bis(*p*-dimethylaminobenzylamino)propane in 15 ml. of methanol was added dropwise 1.1 Gm. (0.015 mole) of isobutyraldehyde during a 5-min. interval. The reaction mixture was refrigerated and the crystalline precipitate which formed was collected and washed with methanol, 5.4 Gm. (92%), m.p. 107–108°. Recrystallization from ethanol provided 4.1 Gm., m.p. 107.3° to 108.3°, for an overall yield of 70%.

Anal.—Calcd. for $C_{25}H_{38}N_4$: N, 14.19. Found: N, 14.48.

The 2-(3,4-dimethoxyphenyl)- derivative was prepared by the following procedure (*B*). To a solution of 9.3 Gm. (0.019 mole) of 1,3-bis(*p*-dimethylaminobenzylamino)propane tetrachloride monohydrate in 190 ml. of water was added dropwise, with stirring, 44 ml. of 10% sodium hydroxide. The precipitated tetramine (III) was collected on a filter, washed well with water, freed of most of the adhering water, then dissolved in 13 ml. of hot methanol. To this hot solution was added a hot solution of 3.1 Gm. (0.019 mole) of veratraldehyde in 6 ml. of methanol. The resulting solution was swirled, cooled to room temperature, then refrigerated. The precipitated derivative was collected and washed with methanol. The dried crude product, 6.3 Gm., was recrystallized from absolute ethanol providing 5.0 Gm. (54%) of white prisms, m.p. 118–119°.

Anal.—Calcd. for $C_{30}H_{40}N_4O_2$: N, 11.47. Found: N, 11.67.

SUMMARY

1. A series of ten 2-substituted-1,3-bis (*p*-dimethylaminobenzyl)hexahydropyrimidines has been prepared.

2. In general, these compounds failed to show any biological properties of significant interest.

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Interaction of Ouabain and Malonate upon the Contractile Response of Myocardium

By DUANE G. WENZEL and IVENS A. SIEGEL

The interaction of two positive inotropic agents, ouabain and malonate, was studied upon the electrically-stimulated rat ventricle strip. Dose-response relationships of each were determined independently and in combination. The response to ouabain was linear within the concentration range tested, while malonate induced depression of the contractile force in higher concentrations. The combined administration resulted in potentiation and it was concluded that ouabain and malonate induce their positive inotropic actions through different mechanisms.

NUMEROUS attempts have been made to determine the mechanism by which the cardiac glycosides increase the efficiency of the failing heart. Such studies have formed the basis for several reviews or summaries (1–3). It has been observed that ouabain, a typical cardiac glycoside, increases the contractile force of the hypodynamic heart when glucose is employed as a substrate, yet does not affect the response in the presence of pyruvate (4). On the basis of this evidence it has been postulated that the cardiac glycosides may remove a rate-limiting step in the glycolytic pathway. Malonate, as metabolic inhibitor with positive inotropic properties, has been observed to have a similar locus of action (5). While the specific site of action within the glycolytic pathway is not known for either ouabain or malonate, it is possible to determine whether they act in the same manner by observing the nature of their interaction upon the myocardial contractile force. Such a study forms the basis of this communication.

EXPERIMENTAL

The method employed to determine the nature of the interaction between ouabain and malonate upon myocardial contractility was the electrically-driven rat ventricle strip. The details of the equipment have been described previously (6). The method, in brief, employed a strip from the right ventricle of young male rats (135–170 Gm.). Such strips were placed in a phosphate-buffered oxygenated medium containing 0.9% sodium chloride, 0.042% potassium chloride, 0.036% calcium chloride, and 1 m*M* sodium phosphate buffer at pH 7.4. The tissue was supramaximally stimulated with 20 v. at a frequency of 4 per sec. Bath volume was 100 ml. and the temperature was maintained at 27°. Drugs were added in 1 ml. of bath solution following withdrawal of an equivalent volume.

Heart strips were allowed to rest in the bath without stimulation for a period of 1.5 to 2 hours, dependent upon the time required for a satisfactory response to stimulation. Following the initiation of stimulation, during what is termed the equilibration period, the amplitude of contraction gradually increased, reaching a maximum termed the 100% level. This was then followed by a steady decline over a period of several hours. Contractile amplitude was measured at 5-min. intervals and drugs were added when it had declined to 75% of the maximal response. In this manner, tests were conducted with the heart strips being in the same relative hypodynamic state.

The method for the determination of the type of interaction between ouabain and malonate is based upon that of Loewe (7). A right isosceles triangle is formed in which the length of each leg represents the concentration of one of the pair of drugs producing the same per cent increase in contractile amplitude. If the concentration of one of the pair is reduced in a stepwise manner from the arbitrary response concentration to nothing, and if this response decrement is compensated for by necessary amounts of the other agent, the plot of the points obtained from these concentration pairs will indicate the type of interaction. If the points lie along the hypotenuse of the triangle, the drugs are additive; if they lie outside the triangle, they are antagonists; and if they lie within the triangle, the drugs are potentiative.

RESULTS

Ouabain was tested for its positive inotropic effect with seven concentrations arrayed in a geometric progression from 9 to 50 $\times 10^{-4}$ m*M*. Four strips were employed for each concentration. As shown in Fig. 1, the response was linear within this concentration range. The increase in contractile amplitude induced by ouabain reached its maximum in 5.8 $\pm$ 0.62 min. for the 28 strips used. After reaching the peak contractile force, the developed tension declined at a faster rate than that noted with untreated strips.

Twelve concentrations of disodium malonate were similarly investigated. The results are also presented in Fig. 1. Only two strips were employed with each of the larger doses of malonate, as indicated by the dotted line. The response of the strips to malonate was distinctly different from that to ouabain. In the lower concentrations (0.26–0.70 m*M*) there occurred a slight immediate decrement (1 to 4%) followed by a slow increase to slightly more than the initial 75% level after 30 to 40 min. With intermediate concentrations (1 to 5 m*M*), the initial decrement was larger (5 to 10%) and was followed by even greater increases in amplitude. The greatest positive inotropic action was developed with a concentration of 5 m*M*. At the highest con-

Received March 26, 1962, from the School of Pharmacy, University of Kansas, Lawrence.
Accepted for publication April 26, 1962.
Supported by a grant from The University of Kansas General Research Fund.

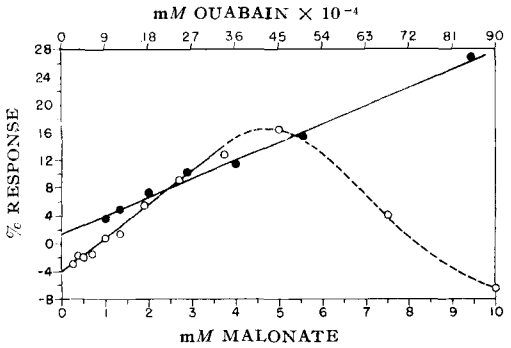


Fig. 1.—Concentration action curves of ouabain and malonate in a substrate-free medium. Ordinate: maximum per cent increase in amplitude (following an initial decrement with malonate). ●, Ouabain; ○, malonate.

centration tested (10 m*M*), a 25% decrement occurred within 1 minute and was followed by a gradual increase which never reached the original (75%) level. All per cent changes were based upon the maximum or 100% amplitude developed after the equilibration period. Those values shown in Fig. 1 are the maximum effects developed after addition of the drug without regard for the time required. With ouabain, the response was maximal in about 6 min. whereas with malonate, this maximum effect required from 30 to 40 min. In order to have the positive inotropic actions of ouabain and malonate coincide when tested for their interaction, malonate was first added to the bath and 30 minutes later, when the response was near maximal, ouabain was added.

The decision as to the per cent increase to be used was based, in part, upon the fact that malonate, in concentrations larger than 5 m*M*, exerted an inhibitory effect. If, for example, the maximum increment induced by 5 m*M* malonate (16%) were selected as the desired positive inotropic action, then any antagonism between the drugs would necessitate using inhibitory concentrations of malonate. An arbitrary 10% positive inotropic action was selected as the desired response. The concentration of each drug required to elicit this 10% positive inotropic action was determined from Fig. 1. For ouabain this was 27.5 × 10^{-4} m*M* and for malonate, 3.0 m*M*.

Five concentrations of ouabain, up to the concentration producing the 10% response (27.5 × 10^{-4} m*M*), were individually tested with sufficient malonate in order to yield the 10% increase in contractile amplitude. The five concentration combinations of ouabain and malonate with the mean values for each are presented in Table I. The curve, or isobol, formed by plotting these equieffective pairs is shown in Fig. 2. From the shape of the isobol it is apparent that the interaction is potentiative.

DISCUSSION

Ouabain was found to produce a positive inotropic action on the rat ventricle strip in concentrations as low as 9 × 10^{-4} m*M* (1:1,500,000). This was considerably below the lowest effective concentration reported by Masuoka and Saunders on the same preparation (8). As these investigators had employed a 50% decrement prior to the addition of the ouabain, in contrast to the 25% reduction in this study, the greater effectiveness could have been related to a lesser depletion of endogenous substrate. Results obtained with disodium malonate were similar to those of Covin and Berman except that they did not observe the decrease in amplitude with concentrations above 5 m*M* (5). There is no obvious explanation for such a difference in the results with high malonate concentrations.

Theoretically, there are at least several possible mechanisms by which drugs can improve the contractility of the myocardium. Energy production, transfer, or utilization may be involved. The contractile protein may be directly affected. Alterations in the electrolyte composition of the myocardium is thought by many to be the underlying mechanism. It is also possible that the efficiency with which the electrical excitation of the cell membrane is coupled with muscle response could be involved. One of the many difficulties related to studying the mode of action of positive inotropic agents is that potential mechanisms are so interdependent that an initiating event is difficult to isolate and identify. In studies employing various

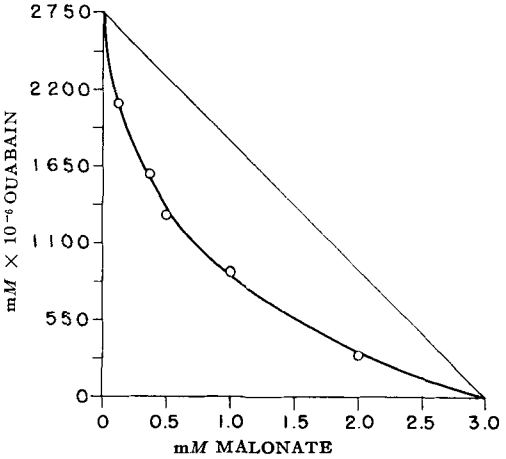


Fig. 2.—Ouabain-malonate isobol. Maximum dose of each produces a 10% increase in amplitude.

TABLE I.—CONCENTRATION PAIRS OF OUABAIN AND MALONATE PRODUCING THE SAME POSITIVE INOTROPIC ACTION[a]

	Concentration, m*M*				
Ouabain × 10^{-2}	0.21	0.16	0.13	0.09	0.03
Malonate	0.26	0.37	0.50	1.0	2.0
Mean positive inotropic action, % ± S.E.	9.99 ± 0.92	10.55 ± 1.07	10.65 ± 0.54	9.62 ± 1.14	10.58 ± 0.87

[a] Per cent positive inotropic actions are the mean values from four determinations.

substrates with a cardiac glycoside or malonate it has been demonstrated that both may act through the removal of a rate-limiting step in the conversion of glycogen or glucose to pyruvate and thus, at least in part, affect energy production or utilization (4, 5). Such an hypothesis does not account for the inhibition observed with high concentrations of malonate, but neither does it preclude the possibility of other concentration-related mechanisms. Malonate may owe a portion of its positive inotropic effect to removal of a rate-limiting step in the glycolytic pathway and possibly to its ability to function as a metabolite (9). Its inhibition of succinic dehydrogenase (10) could account for the initial decrement and that decrement observed with high concentrations. While the results of the ouabain-malonate interaction suggest neither the sites of action nor the mechanisms, they do indicate the individual actions to be different from one another. If these agents shared the same site or mechanism of action, the interaction would be either additive or antagonistic. The potentiative response indicates complementary activity.

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Stability of Vitamins A, B_1, and C in Selected Vehicle Matrices

By KEE-NENG WAI, H. GEORGE DEKAY, and GILBERT S. BANKER

The relative stability of vitamins A, B_1, and C in eight commonly used solid vehicle matrices was determined. Vitamin preparations, each containing one vitamin and one solid matrix, were first subjected to accelerated aging tests and were evaluated for their loss in vitamin contents. The results indicated that a diluent moisture content in excess of one per cent played an important role in determining the stability of these preparations. Mannitol and lactose were found to be superior solid diluents on the basis of the stability studies conducted. The influence of formulation (use of coated or U.S.P.-grade vitamins), method of combination, and manufacturing procedures were studied for their effect on vitamin stability in an uncoated tablet dosage form.

IN RECENT years many new, uncoated multivitamin tablets have appeared on the market. The majority of these products have been chewable, pediatric vitamin tablets, although conventional vitamin tablet formulations in uncoated, compression-coated, and film-coated form are also making their appearance. The advantages of uncoated vitamin tablets over vitamin liquids, capsules, and coated tablets are numerous, obvious, and of considerable economic significance. The development of commercially feasible uncoated vitamin tablet formulations is being made possible by at least three advances: (*a*) the application of new excipient materials to solid vitamin products; (*b*) the commercial availability of new, more stable chemical forms of vitamins, and coated, wax imbedded, and other modifications of vitamin materials, and (*c*) studies which accurately describe the stability, reaction kinetics, and incompatibilities of the various vitamins.

Received March 29, 1962, from Purdue University, School of Pharmacy, Lafayette, Ind.
Accepted for publication May 24, 1962.
Abstracted from a thesis submitted to the Graduate School of Purdue University, by K. N. Wai in partial fulfillment of the requirements for the Master of Science degree.
Presented to the Scientific Section, A.PH.A. Las Vegas meeting, March 1962.

The reports of McLaughlan, Clark, Campbell, and McLeod (1–3) have revealed that vitamins A, B_1, B_{12}, and pantothenic acid in many commercial multivitamin products are quite unstable. Ascorbic acid is known to be incompatible with riboflavin, vitamin B_{12}, and folic acid (4). Thiamine reacts with riboflavin (5) and folic acid (6). Extensive investigations of the interactions among thiamine, niacinamide, and vitamin B_{12} have also been carried out in the past few years (7–14).

Accelerated aging tests of vitamin products have been conducted by Garrett (15) and Yamamoto (16). These workers related the stability of various vitamins with storage time and temperature and found that the degradation of ascorbic acid, thiamine, vitamin B_{12}, and pantothenyl alcohol was a first-order reaction and the degradation rate of vitamin A was independent of concentration. They also showed that at 45° the degradation of vitamin A in 8 weeks and ascorbic acid in 6 weeks was approximately equivalent to their degradation at room temperature for 1 year. Taub and Katz (17) have indicated that thiamine decomposition in tablets and capsules, after storage at 45°

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for 3 weeks, was comparable to a year of shelf life.

While a good deal has been learned about vitamin stability in recent years, no comprehensive report of the stability of the less stable vitamins in common, solid vehicles is in the literature. Such information could be of value in the development of uncoated tablets, whether designed to be chewed or swallowed, compression-coated tablets in which a portion of the vitamin formulation is in the coat or all the vitamin formulation is in the coat and the core is comprised of minerals, or film-coated tablets in which the film adds pharmaceutical elegance but affords incomplete protection to oxygen or water vapor transmission.

It was the objective of this research to study the stability of three selected, relatively unstable, vitamins in different solid vehicle matrices and to study various formulations and manufacturing procedures which might relate to the stability of solid multivitamin products.

EXPERIMENTAL

Materials.—The vitamins A and D[1] used in this study were in the form of a granulation containing gelatin, sugar, and starch with a labeled content of 500,000 U.S.P. units (150 mg.) of vitamin A acetate and 50,000 U.S.P. units of vitamin D_2 per gram of granulation. The other vitamins used were U.S.P.-grade chemicals or coated vitamins in the form of thiamine mononitrate Mercote,[2] niacinamide Mercote, riboflavin Mercote, and ascorbic acid coated with 5% ethylcellulose type N.[3]

The diluents used included mannitol, lactose, sucrose, aluminum hydroxide dried gel, kaolin, starch, calcium sulfate, and hydrous dextrose. All of these were U.S.P.- or N.F.-grade chemicals with the exception of the calcium sulfate which was in the form of $CaSO_4 \cdot 2H_2O$.[4] The lubricant in all of the preparations was magnesium stearate, U.S.P.

Equipment.—The equipment used to prepare the solid vitamin products included a kitchen-aid model Hobart mixer[5] for the mixing of powders and granulations; stainless steel wire screens for the reduction of particle size and mixing of powders (30 mesh) and for granulating and resizing (20 mesh); the Stokes oscillating granulator,[6] fitted with a 12-mesh screen, for dry granulating; and a Stokes B2 rotary tablet machine[6] equipped with $^3/_8$-inch standard concave punches and dies and $^5/_8$-inch flat-face punches and dies, for tableting and slugging, respectively.

The instruments used for the assays of the vitamins included a Beckman model DU spectrophotometer[7] for the measurement of absorbance of vitamin A solution and a Fisher nefluorophotometer[8] for the measurement of the fluorescence of thiochrome.

A constant-temperature electric oven, adjusted to 45° (±2°) was used to store the tablets for the accelerated aging tests. The other equipment used included the Monsanto hardness tester[9] and the Cenco moisture balance.[10]

Methods of Assay for Vitamins A, B_1, and C.—The official methods for the assay of these three vitamins as described in the monograph of decavitamin tablets U.S.P. were used throughout the course of this study. However, some modifications were made. In the assay of vitamin A, the saponification of vitamin A acetate was carried out in 7 ml. of glycerin, 25 ml. of alcohol, and 3 ml. of 50% potassium hydroxide solution. The glycerin was used because it had a greater softening effect than alcohol on the brand of commercial vitamin A granules used. All calculations for vitamin A content were based on the absorbance at 325 mμ instead of the three points prescribed by the U.S.P. In the assay of vitamin B_1, the concentration of the "Standard Preparation" was about 1.0 mcg. per ml. instead of 0.2 mcg. per ml. as suggested in the U.S.P. This change was made in order to permit the measurement of fluorescence at the optimum sensitivity range of the equipment employed in this work. The alcoholic extracts of the vitamin A and aqueous extracts of vitamin B_1 were transferred into 100-ml. volumetric flasks and brought to volume with dilute alcohol or water before filtration. Exactly 50 ml. of the filtrates was collected for the assays.

Study of the Stability of Vitamins A, B_1, and C in Solid Matrices.—Thirty-six samples, each containing one vitamin, one diluent, and 0.5% magnesium stearate, were separately prepared with the three vitamins and 12 commonly used solid matrices. The solid matrices included mannitol, sucrose, lactose, calcium sulfate, aluminum hydroxide dried gel, starch, kaolin, dextrose, and combinations of 80% mannitol with 20% each of starch, aluminum hydroxide dried gel, sucrose and dextrose. Each gram of sample contained 4 mg. of vitamin A in the form of dry vitamin A acetate with D, or 5 mg. of thiamine hydrochloride, U.S.P., or 300 mg. of ascorbic acid U.S.P. These combinations were compressed on a rotary-tablet machine into one-gram disks, $^5/_8$ inch in diameter. These disks were employed to individually study the stability of the vitamins in the various matrices in a compacted, simulated tablet condition. They were kept in six-ounce, brown, dry-square bottles and stored at 45° and at room temperature. The vitamins in these preparations were assayed as follows: (*a*) prior to storage, (*b*) after 1 month's storage at 45°, (*c*) after 3 months' storage at room temperature, and (*d*) after 3 months' storage at 45°.

Study of the Stability of Multivitamin Chewable Tablets.—Following the study of vitamin stability in various solid matrices, one of the tablet diluents was selected for subsequent research dealing with the stability of multivitamin chewable tablets. Utilizing mannitol as the selected diluent, six batches of tablets were prepared. These tablets differed

[1] Hoffmann-La Roche, Inc., Nutley, N. J.
[2] Merck & Co., Inc., Rahway, N. J.
[3] Hercules Powder Company, Wilmington, Del.
[4] Miles Laboratory, Inc., Elkhart, Ind.
[5] Hobart Manufacturing Co., Troy, Ohio.
[6] F. J. Stokes Corporation, Philadelphia, Pa.
[7] Beckman Instruments, Inc., Fullerton, Calif.
[8] Fisher Scientific Company, New York, N. Y.
[9] Monsanto Chemicals, St. Louis 4, Mo.
[10] Central Scientific Company, Chicago, Ill.

TABLE I.—A FORMULA FOR A MULTIVITAMIN CHEWABLE TABLET (TABLET NO. 1)

Ingredients	Quantity in Gm.	Labeled Content per Tablet	Overage, %
Granulation 1			
Vitamin A } granules	11.00	5000 Units	10
Vitamin D }		500 Units	10
Thiamine hydrochloride	1.65	1.5 mg.	10
Mannitol	94.70	...	...
Magnesium stearate	0.54	...	...
Granulation 2			
Ascorbic acid	66.00	60 mg.	10
Mannitol	189.50	...	...
Magnesium stearate	1.28	...	...
Granulation 3			
Niacinamide	10.50	10 mg.	5
Riboflavin	1.10	1 mg.	10
Cobalamin concentrate (1:1000)	2.20	2 mg.	10
Pyridoxine hydrochloride	2.20	2 mg.	10
Calcium pantothenate	4.50	3 mg.	50
Mannitol	94.70	...	...
Magnesium stearate	0.57	...	...
Running powder and flavors			
Magnesium stearate	2.60	...	...
Orange-dry flavor	5.00	...	...
Pineapple-dry flavor	5.00	...	...
Saccharin	7.50	...	...
To make 1000 tablets			

from one another in the forms of vitamins used, the methods of vitamin combination, and the method of manufacture. All of these formulas contained equal labeled quantities and overages of the vitamins. To prepare the tablets, the vitamins which were known to be mutually incompatible were granulated separately with a part of the mannitol and lubricated with magnesium stearate. One of the typical working formulas is given in Table I and the composition and methods of manufacture of the six batches of tablets are summarized in Table II.

These multivitamin chewable tablets were assayed immediately after manufacture and after storage for 1 month at 45°. Three commercial chewable tablets were studied simultaneously for the purpose of comparison. The hardness of these tablets and any changes in physical appearance with aging were also observed.

RESULTS AND DISCUSSION

The moisture contents of the solid vehicles used in this work are shown in Table III.

TABLE III.—MOISTURE CONTENT OF SOLID VEHICLES STUDIED

Vehicle	Moisture, %[a]
Mannitol	0.2
Sucrose	0.2
Lactose	1.0
Aluminum hydroxide	15.0
Starch	8.0
Calcium sulfate	2.7
Kaolin	1.0
Dextrose	8.0
Mannitol and sucrose	0.2
Mannitol and aluminum hydroxide	2.9
Mannitol and starch	1.8
Mannitol and dextrose	1.6

[a] Moisture contents were determined using the Cenco moisture balance at a pan temperature of 90°.

Tables IV, V, and VI show that vitamin A was most stable in manitol and lactose; thiamine was stable in mannitol, sucrose, lactose, and kaolin; and ascorbic acid appeared to have good stability in all of the diluents with the exception of aluminum hydroxide. Most of the ascorbic acid preparations, however, changed color during the storage period. The color change was not universally indicative of a loss of vitamin potency. Mannitol, sucrose, and lactose were the only three diluents in which no color change was observed with ascorbic acid under the conditions of storage.

Table IV also shows that the degradation of vitamin A at 45° for 1 month was usually slightly greater than its degradation after storage at room temperature for 3 months. Accordingly, it is expected that the degradation of vitamin A at 45° for 3 months might be approximately equal to its degradation at room temperature for a year.

Thiamine appears to be much more thermolabile than vitamin A. The accelerated aging test of the vitamin B_1 preparations after 1 month clearly indicated the role of the diluent on the stability of the vitamin.

The degradation of vitamins A, B_1, and C is accelerated by the presence of moisture in the solid vehicles. In mannitol, sucrose, lactose, and kaolin, which contained not more than 1% moisture, these vitamins were quite stable. In starch, aluminum hydroxide, and dextrose which contained 8, 15, and

TABLE II.—DESIGNATION OF TABLET COMPOSITION AND METHOD OF MANUFACTURING

Tablet Number	Form of Vitamins	Granulation Method	Granulation Agent	Vitamin-Granulation Combinations 1[b]	2	3
1	USP Powder	Dry	...	A, D, & B_1	C	Other B Vit.
2	Coated[a]	Dry	...	A, D, & B_1	C	Other B Vit.
3	Coated	Dry	...	A & D	B_1 & C	Other B Vit.
4	Coated	Wet	Alcohol	A & D	B_1 & C	Other B Vit.
5	Coated	Wet	Dil. Alc.	A & D	B_1 & C	Other B Vit.
6	Coated	Wet	Water	A & D	B_1 & C	Other B Vit.

[a] The coated vitamins included the Mercotes and the ethylcellulose coated ascorbic acid. The other B vitamins in the formula were uncoated U.S.P. grade. Since the coated vitamins contained less active ingredient than an equal weight of U.S.P.-grade vitamin, a correspondingly larger amount of coated vitamin was taken and the weight of the diluent in these formulations was reduced so that each tablet at the same theoretical weight would contain the same potency of each vitamin.
[b] The vitamins A and D in formulas 3 to 6 were not granulated with the diluent. The vitamin granules were added directly to granulations 2 and 3.

TABLE IV.—STABILITY OF VITAMIN A IN SOLID VEHICLE MATRICES

Vehicle	Initial Activity, mg./Gm.	Activity Retained after Storage: 30 Days at 45°C. mg./Gm.	%	90 Days at 45°C. mg./Gm.	%	90 Days at Room Temp. mg./Gm.	%	Color after Storage
Mannitol	4.16	3.75	90	3.06	74	...	...	white
Sucrose	3.97	3.18	80	2.65	67	...	...	white
Lactose	4.08	3.63	89	3.09	76	3.47	85	white
Calcium sulfate	3.67	2.75	75	2.43	66	2.92	80	white
Aluminum hydroxide	4.03	2.95	73	2.29	56	...	...	brown
Starch	3.99	2.37	59	2.00	50	3.37	85	yellow
Kaolin	3.60	2.71	75	2.42	67	...	...	brown
Dextrose	4.08	3.32	81	2.75	67	...	...	buff
Mannitol and sucrose	3.77	3.33	88	2.73	72	3.40	90	white
Mannitol and aluminum hydroxide	3.75	2.26	60	2.25	60	3.30	88	brown
Mannitol and starch	3.86	2.71	70	2.38	62	3.24	84	buff
Mannitol and dextrose	3.87	3.21	83	2.77	72	...	...	buff

TABLE V.—STABILITY OF VITAMIN B_1 (U.S.P. POWDER) IN SOLID VEHICLE MATRICES

Vehicle	Initial Activity, mg./Gm.	Activity after Storage: 30 Days at 45°C. mg./Gm.	%	90 Days at Room Temp. mg./Gm.	%	Color after Storage
Mannitol	5.43	5.42	100	5.30	98	white
Sucrose	5.16	5.21	100	5.12	99	white
Lactose	5.74	5.79	100	...	...	white
Calcium sulfate	5.61	4.94	88	5.58	99	white
Aluminum hydroxide	5.75	2.63	46	5.65	98	white
Starch	5.12	0.91	18	5.00	98	white
Kaolin	4.07	4.05	100	4.20	100	brown
Dextrose	4.85	0.63	13	4.24	87	white
Mannitol and sucrose	4.68	4.50	96	4.42	94	white
Mannitol and aluminum hydroxide	5.38	1.55	29	5.01	93	white
Mannitol and starch	5.46	1.73	32	4.98	91	white
Mannitol and dextrose	5.05	1.02	20	4.45	88	white
Mannitol (and coated vitamin B_1)	6.13	6.14	100	...	...	white

TABLE VI.—STABILITY OF VITAMIN C (U.S.P. GRANULAR) IN SOLID VEHICLE MATRICES

Vehicle	Initial Activity, mg./Gm.	Activity after Storage for 90 Days at 45° C. mg./Gm.	%	Color after Storage for 90 Days at 45°C.
Mannitol	289	290	100	white
Sucrose	282	279	99	white
	192	205	100	white
Lactose	307	298	97	white
	202	200	99	white
Aluminum	282	187	66	brown
hydroxide	212	156	74	brown
Starch	310	315	100	yellow
	207	204	99	yellow
Calcium	293	291	99	buff
sulfate	206	209	100	buff
Kaolin	285	287	100	brown
	191	181	95	brown
Dextrose	282	289	100	straw
	206	196	95	straw
Mannitol and sucrose	292	281	96	white
Mannitol and aluminum hydroxide	299	296	99	brown
Mannitol and starch	301	296	98	buff
Mannitol and dextrose	296	295	100	yellow

8% moisture, respectively, the vitamins were generally very unstable.

It is believed that kaolin adsorbed a part of the thiamine and caused the apparent low activity of thiamine in this combination. Also, in the assays of vitamin A in lactose and glucose, excessive caramelization of the sugars was observed. The light absorbance of the isopropanol extracts of these preparations measured at wavelengths of 310 mμ, 325 mμ, and 334 mμ indicated the presence of interference materials. However, no correction was made for this interference since all data were calculated by the same method according to the light absorbance of the sample at 325 mμ.

Table VII shows that maximum stability was achieved in Tablet No. 2 by using the dry granulation method and by granulating vitamins A and B_1 separately from the other vitamins. Tablets No. 3, 4, 5, and 6 each showed approximately the same degree of stability, but tablets No. 5 and 6, granulated with dilute alcohol and water, respectively, changed color quite rapidly. The tablets prepared by double compression had no apparent change in color after storage at 45° for a month and were generally softer than those prepared by the wet-granulation method. These results indicated that the use of water as the granulating agent or granulating agents containing water caused a more rapid change in the color of the solid multivitamin products than when no water was added. The findings

TABLE VII.—STABILITY OF VITAMINS A, B_1, AND C IN MULTIVITAMIN CHEWABLE TABLETS

Tablet[a] Number	Hardness, Kg.	Color Stability	Vitamins	Labeled Content, mg./Gm.	Initial Activity, mg./Gm.	Activity after Storage for 30 Days at 45°C. mg./Gm.	%
1	2.2	stable[c]	A	3.0	2.96	2.70	91.4
			B_1	3.0	3.43	3.38	98.5
			C	120	136.0	136.5	100
2	3.1	stable	A	3.0	2.96	2.81	95.1
			B_1	3.0	4.03	3.87	96.0
			C	120	132.6	135.2	100
3	3.3	stable	A	3.0	3.43	3.06	89.2
			B_1	3.0	3.99	3.92	98.2
			C	120	138.8	137.4	99.0
4	6.5	stable	A	3.0	3.58	3.16	88.3
			B_1	3.0	3.98	3.86	97.0
			C	120	135.8	136.5	100
5	8.1	darkened slowly	A	3.0	3.28	2.84	87.7
			B_1	3.0	3.95	3.85	97.2
			C	120	134.4	132.9	98.9
6	7.3	darkened	A	3.0	3.48	3.06	88.0
			B_1	3.0	3.89	3.89	100
			C	120	134.0	132.8	99.1
7	3.1	darkened slowly	A	2.75	3.61	2.97	93.9
			B_1	5.5	20.55	18.22	88.7
			C	92	104.7	99.5	95.0
8[b]	8.0	darkened slowly	A	3.20	3.46	4.04	92.7
			B_1	2.6	3.58	3.23	90.2
			C	123	146.9	148.6	100
9[b]	3.2	darkened slowly	A	4.35	4.82	4.26	88.5
			B_1	5.8	7.03	6.98	99.3
			C	172	187.4	184.0	98.2

[a] See Table II. [b] A commercial multivitamin chewable tablet. [c] "Stable" means no apparent change in color during the period of accelerated aging test.

of Table VII also showed that the manner in which the vitamins were combined was as important as the granulating method used (wet or dry) or the granulating agent used.

CONCLUSIONS

Of the eight commonly used diluents evaluated, mannitol appeared to be the best diluent with regard to the stability of the vitamin products studied with lactose being a close second. A diluent moisture content in excess of 1% played an important part in catalyzing the degradation of vitamins B_1 and C. Some chemical change in the tablets containing ascorbic acid is manifested by an intense discoloration of the products, although the actual assay values may not be significantly reduced. Hence, the choice of a nonhygroscopic solid vehicle is essential in formulating a stable, uncoated, multivitamin tablet.

The stability of uncoated multivitamin tablets was found to be improved by the following techniques:

1. Use of coated ascorbic acid, thiamine, riboflavin, and niacinamide. This process enhances the stability of the vitamins.

2. Preparation of the granulations of the incompatible vitamins separately with a portion of the inert matrices. The best stability results were obtained when the vitamins A, D, and B_1 were granulated with one-fourth of the matrix, the vitamin C with one-half of the matrix, and the remaining B vitamins with one-fourth of the matrix. These three granulations were blended together in the proper proportion before compression.

3. Avoiding use of water as a granulating agent. Vitamin tablets granulated with water or dilute alcohol discolored rather rapidly, however the assays of these tablets indicated no significant difference in stability from those granulated with alcohol or prepared by the dry-slugging method.

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Experimental Method for Studying Growth Rates of Particles in Aqueous Media

By W. I. HIGUCHI and P. K. LAU

A simple procedure for following growth of suspension particles has been developed. It is suitable for studying mechanisms of growth, for evaluation of potential growth retarding agents, and possibly for accelerated stability studies in dispersed systems. It involves the determination of the particle concentration and size distribution as a function of time with the Coulter counter. The sensitivity of the method is better than 0.1 μ for particles in the micron size range. Prior removal of the particles from the suspension is unnecessary. The technique has been applied to growth rate studies in the methylprednisolone system.

THERE ARE A NUMBER of situations in which an understanding of the factors determining the rate of crystal growth is helpful to the pharmacist. In connection with stability, the coarsening of suspensions by growth of the larger particles at the expense of the smaller ones is a good example. The problem of caking, the cementing together of particles in a sediment, is also well known. More recently, attention (1) has been given to the possible utilization of more energetic crystal forms of drugs in pharmaceutical preparations. The problem of preventing the conversion of these crystals may be frequently one of inhibiting the growth of more stable and less active forms. Thus, a systematic investigation of the growth rates of crystals may greatly extend our knowledge in these areas.

To study situations mentioned above, the experimental method should be one in which the following requirements are met: The method should be highly sensitive, capable of detecting growth in the order of one micron. The method should be reproducible or, alternatively, statistically significant. This requirement is most conveniently met if the growth of a large number of crystals could be followed at the same time. Finally, the method should be convenient and rapid if reasonable progress is to be expected.

The present report describes a method which meets these requirements. It is highly sensitive, reproducible, and simple. In its present form it might be used, for example, to perform accelerated stability studies and to screen and study potential growth inhibiting agents.

EXPERIMENTAL

General Consideration.—It was decided to select a method which involves following the changes in the distribution of suspension particle sizes with time. A solution suitably supersaturated with a drug could be seeded with crystals of the drug and the size distribution of the crystals determined from time to time with the Coulter counter.[1] If, during the experiment, sedimentation or aggregation of the crystals remains negligible, the changes in the particle sizes may be attributed entirely to growth by molecular transport and deposition.

The steroid, methylprednisolone, was chosen for this study because the polymorphism of this compound has been investigated (2). In the present studies growth from solution of the most stable form of methylprednisolone was followed.

Procedure.—Methylprednisolone[2] was recrystallized from a 50% ethanol-water solution and dried. The crystals were then finely ground by means of a mortar and pestle. An amount of the powder equal to about four times that necessary for saturation was placed in 500 to 1000 ml. of redistilled water in a round-bottom flask. The suspension was stirred vigorously for at least 2 hours at some temperature higher than the 25° employed for the growth studies. In most of the experiments this saturation temperature was around 50° where the solubilities are about twice that at room temperature. After saturation, the suspension was filtered through a 0.45 μ pore-size Millipore[3] filter and the filtrate was placed in a 1 L. glass-stoppered round-bottom flask, ready for seeding.

The seed crystals were prepared and added to the supersaturated solution in the following manner: About 10 ml. of the above prepared solution was placed in a 25-ml. glass-stoppered graduated cylinder. To this was added about 25 mg. of the finely ground drug powder. To promote dispersion, surfactant was added, and the seed suspension was subjected to about 5 minutes of ultrasonic irradiation in a cleaning unit.[4] Then it was allowed to stand undisturbed for about 30 minutes to 2 hours to insure removal of large crystals by sedimentation. After this period, a few tenths of a ml. was pipetted from the uppermost regions of this suspension and added to the supersaturated solution.

The flask containing the seeded solution was then placed in a water bath thermostated at 25.0 ± 0.05°. A Teflon-coated magnetic stirrer activated from below the bath provided gentle agitation of the suspension. Periodically, 50 ml. of the growing

Received March 27, 1962 from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication June 13, 1962.
Supported by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

[1] Marketed by Coulter Industrial Sales, Chicago, Ill.
[2] Kindly supplied to us by Dr. J. W. Shell, The Upjohn Co., Kalamazoo, Mich.
[3] Marketed by Millipore Filter Corporation, Bedford, Mass.
[4] Model DR-125AH, Acoustica Associates, Mineola, N. Y

suspension was pipetted out and mixed with an equal volume of 0.9% sodium chloride solution which had been previously saturated at 25° with methylprednisolone and filtered through the 0.45 μ Millipore filter. This mixing with an electrolyte was necessary for the Coulter counter operation. It also reduced the supersaturation, thus retarding growth during the actual counting.

In all of the present experiments, the 50- and the 100-μ aperture tubes were employed. With these, the 1- to 20-μ diameter (equivalent volume sphere) range of particle sizes could be conveniently studied. The 100-μ aperture tube was calibrated with a suspension of partially aggregated 1.83 μ polystyrene latex particles which had been sized with the 50-μ aperture tube. The latter was calibrated earlier (3, 4) with nonaggregated 1.17 μ and 1.83 μ polystyrene latex particles.

There were some important variations in the above procedure. In most experiments a surfactant was added to the growth solution either during the high temperature saturation period or after filtering of the suspension. In some experiments the suspension equilibrated at high temperatures was not filtered through the Millipore filter but instead through a medium-grade sintered glass filter. In these runs, the solutions were not seeded since it was expected that much of the fine crystals would pass through the filter. However, this procedure was unsatisfactory in that the crystallite population varied greatly from run to run. In another modification of the procedure, about 0.1% of Tween 80 was added to the 0.9% sodium chloride solut on which was used to mix with the aliquot of the growing suspension for counting. The presence of this surfactant apparently helped to further reduce growth during the counting.

Along with the Coulter counter determinations, the solution concentrations of the drug were determined as a function of time. Samples were removed from the growth flask by means of a syringe fitted with a Swinny adapter[5] for a Millipore filter. The solutions were then spectrophotometrically assayed after appropriate dilution in 95% ethanol.

DISCUSSION OF THEORY AND EXPERIMENTS

Change of Particle Size with Time (Theory).—Before we proceed with the presentation of data, let us briefly consider the mathematical relationships which apply to the various models for particle growth. These will be helpful in interpreting the experimental results.

For the growth rate, G_i, of a given particle of radius (equivalent volume sphere), a_i, n the distribution we may write

$$G_i = 4\pi\rho a_i^2 \frac{da_i}{dt} \qquad \text{(Eq. 1)}$$

where t is time and ρ is the density of the solid drug. This may be combined with various rate expressions presented below to give a relationship between a_i and t.

If the growth rate is proportional to the particle radius, then

$$G_i = K_1 a_i \qquad \text{(Eq. 2)}$$

where K_1 is a constant if everything else but a_i is constant. This rate law applies to the important case of the diffusion-controlled growth of fine isometric particles ($\lesssim 10$ μ) under zero to moderate agitation (5). For this special case, K_1 may be expressed as

$$K_1 = 4\pi D\Delta C \qquad \text{(Eq. 3)}$$

where D is the diffusion coefficient of the drug molecule and ΔC is the supersaturation defined as $\Delta C = C - C_o$ where C is the solution concentration of the drug and C_o is the solubility. If Eq. 2 and 1 are combined, we obtain

$$\frac{da_i}{dt} = \frac{K_1}{4\pi\rho a_i} \qquad \text{(Eq. 4)}$$

Thus, according to this equation, the rate of change of the particle radius (or diameter) with time is proportional to the reciprocal of the radius.

Consider now the case in which the growth rate is proportional to the square of the particle radius, *viz.*

$$G_i = K_2 a_i^2 \qquad \text{(Eq. 5)}$$

where again K_2 is a constant if everything but a_i is constant. Equation 5 may apply to the growth of isometric particles in which the rate of growth is controlled by the particle surface area and the probability of growth is the same for all surfaces of the particle. If Eq. 5 is combined with Eq. 1 we obtain

$$\frac{da_i}{dt} = \frac{K_2}{4\pi\rho} \qquad \text{(Eq. 6)}$$

This relationship predicts that the rate of change of a_i with time is constant. As will be seen later, most of the data appear to fit Eq. 6 the best.

The last case we shall present is that in which the growth rate is proportional to the cube of the radius of the equivalent volume sphere. We may write

$$G_i = K_3 a_1^3 \qquad \text{(Eq. 7)}$$

where again K_3 is a constant if a_i is the only variable. To the authors' knowledge, no simple physical counterpart exists for this case. The following case, however, might approximate the cube law. If the particles in the initial distribution are composed of aggregates of relatively uniformly sized primary particles, and if the growth rate is surface-area controlled, then at constant t Eq. 7 might apply since the true surface area would be proportional to the mass in this instance. If Eq. 7 is combined with Eq. 1, we obtain

$$\frac{da_i}{dt} = \frac{K_3 a_i}{4\pi\rho} \qquad \text{(Eq. 8)}$$

Thus, for this case the rate of change in a_i is proportional to a_i.

Experimental Results.—The results of experiments under a number of different conditions are presented in Figs. 1–5. These are illustrative of the method. In each, both the Coulter counter data and the solution concentrations of the drug are given as a function of time. The counter data are plotted as the cumulative count *vs.* particle size. Note that the derivatives of these plots would be the usual particle-size distribution curves.

[5] Marketed by Millipore Filter Corporation, Bedford, Mass.

In all of these results the time decrease in drug concentration of the solutions agreed with the amount of growth calculated from the counter data. The calculations here involve the use of the following equation for the conservation of matter

$$\int_{a=o}^{\infty} {}^4/_3\pi\rho\, a^3 n(a)\, da = \text{constant} - M$$

where M is the solution concentration of the drug and n (a) is the distribution function and equal to minus twice the slope of the curves in the plots. The integral may be easily evaluated at various times by means of conventional numerical methods (6) and compared with the right side of this equation. The data given in Fig. 1 may be used to best illustrate this, since in this instance, appreciable solution depletion occurred. This mass balance is a confirmation of the validity of the method.

In cases where all of the initial seed particles had grown beyond the lower size limit of the counter, plateaus or near-plateaus appeared in the plots of counts *vs.* size (see Figs. 1 and 5). This again demonstrated that growth is the main contribution to the time change in the particle size distribution. If, say, aggregation were to be important in the early stages of the experiment, the development of a plateau would be highly unlikely. Instead, the slope of the plots would be expected to steadily increase with decreasing particle diameter.

In those runs in which plateaus appeared, the levels of the plateaus remained constant for an appreciable length of time. For example, in the 0.025% Tergitol 4 case (Fig. 1) the level of the plateau remained constant to a few per cent for about 2 hours during which rapid growth took place. However, after this period, the plateau level began to fall slowly, then more rapidly. This suggested that when the number of relatively large particles reached some level, aggregation or wall losses became important. This drop-off of the plateau level occurred in all experiments whenever the number of large particles became great.

Negligible aggregation and wall losses in the absence of large particles was also demonstrated by an experiment identical to that shown in Fig. 1, except that the supersaturation was about 0.03

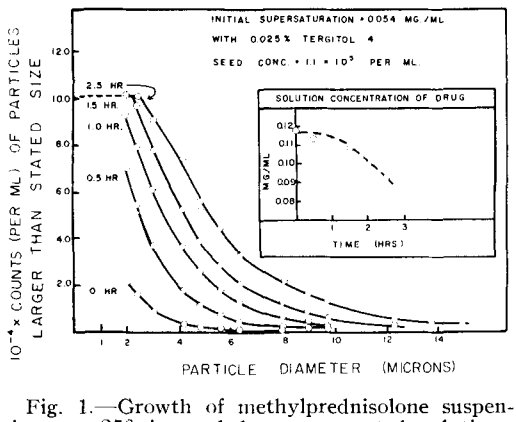


Fig. 1.—Growth of methylprednisolone suspensions at 25° in seeded supersaturated solutions. Main plots are number integral size distribution as function of time. Insets give data on relief of supersaturation over same time ranges.

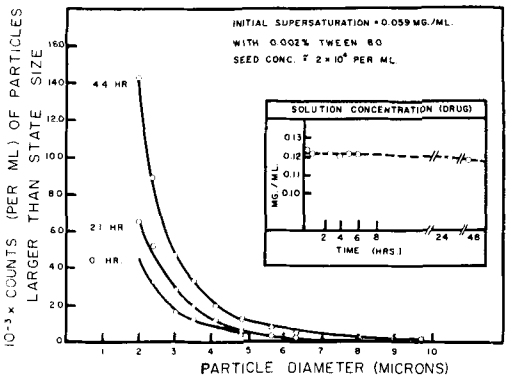


Fig. 2.—Growth of methylprednisolone suspensions at 25° in seeded supersaturated solutions. Main plots are number integral size distribution as function of time. Insets give data on relief of supersaturation over same time ranges.

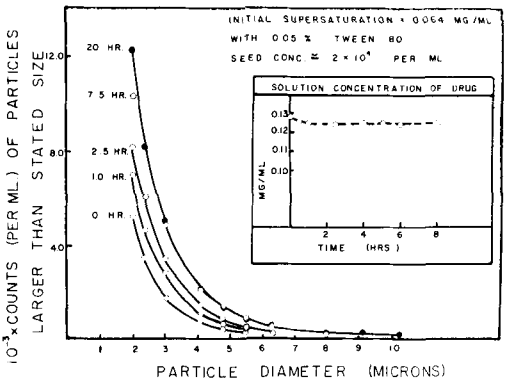


Fig. 3.—Growth of methylprednisolone suspensions at 25° in seeded supersaturated solutions. Main plots are number integral size distribution as function of time. Insets give data on relief of supersaturation over same time ranges.

mg./ml. The growth rate was found to be negligible in this case and both the particle concentration and the distribution of sizes remained unchanged for the duration of the experiment (about 5 hr.). Thus, aggregation or wall losses was absent when large particles were absent.

Let us now direct our attention to the actual rates of growth and the rather interesting effects of small amounts of surfactants. When no surfactant was employed (see Fig. 5) the rate of growth was most rapid. In fact, from the data given in Fig. 5, the rate was found to be within a factor of approximately three of the diffusion controlled rate predicted by Eqs. 3 and 4 and with $D = 5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ for methylprednisolone. The addition of one drop of Tween 80 (see Fig. 2) reduced the rate of growth by about 500-fold. At somewhat higher Tween 80 concentrations (see Fig. 3) there appeared to be an initial period of growth; this was followed by a great reduction in the rate. In the presence of 0.05% Triton X-100, similar effects were observed (Fig. 4). Tergitol 4, an anionic surfactant, did not reduce the rate (Fig. 1) as markedly as the nonionics. Comparable amounts of sodium lauryl sul-

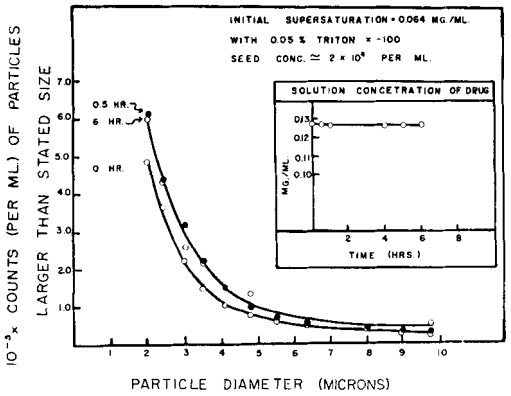


Fig. 4.—Growth of methylprednisolone suspensions at 25° in seeded supersaturated solutions. Main plots are number integral size distribution as function of time. Insets give data on relief of supersaturation over same time ranges.

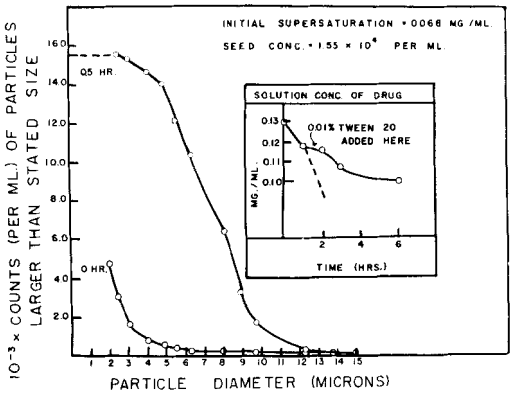


Fig. 5.—Growth of methylprednisolone suspensions at 25° in seeded supersaturated solutions. Main plots are number integral size distribution as function of time. Insets give data on relief of supersaturation over same time ranges.

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In most instances, particularly for those cases in which the growth rates were relatively small, the rate law given by Eq. 6 appeared to be approximately obeyed. In terms of the plots of the cumulative counts *vs.* particle size, conformation of the data to Eq. 6 would mean that the curves would be displaced to the right at a constant rate with respect to time. The results shown in Figs. 2 and 3 appear to do just that. This suggests strongly that in these cases the growth rates are proportional to the surface area of the particles.

In the experiments with Tergitol 4, it was observed that, while a given ordinate value moved constantly with time to the right, the upper portions of the curves moved more slowly than the lower. For reasons mentioned earlier, this was probably not due to aggregation of the particles. This type of behavior appears to be analogous to the theoretical case leading to Eq. 8. At any given time t the smaller particles appear to be growing more slowly than the larger ones. Further studies are necessary to establish the situation unambiguously for this type of growth behavior.

REFERENCES

(1) Higuchi, T., Second Annual National Industrial Pharmaceutical Conference, Land O' Lakes, Wisconsin, June 1960. See also, Higuchi, T., and Shefter, E., "Influence of Hydrate and Solvate Formation on Rates of Solution and Solubility of Crystalline Drugs," preprints of symposium papers, Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

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A technique is described whereby urine can be excreted by human subjects, at a pH near 5.0 or 8.0, with a variation of not more than 0.1 or 0.2 pH unit over periods of at least 15 hours. The significance of controlled urinary pH in humans with respect to the excretion kinetics of a weak acid, sulfaethidole, has been determined. The harmonic mean $t_{1/2}$ for SETD at a controlled urinary pH of 5.0 is 11.4 hours; the $t_{1/2}$ for this drug at a controlled urinary pH of 8.0 is 4.2 hours.

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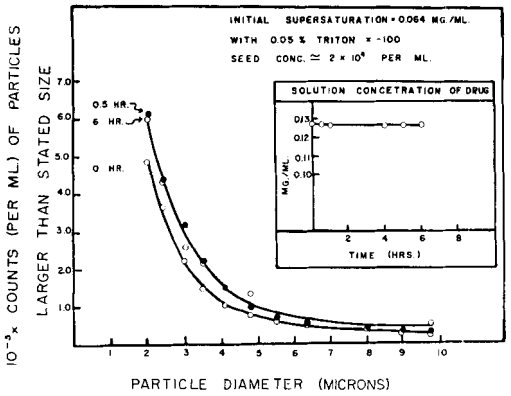


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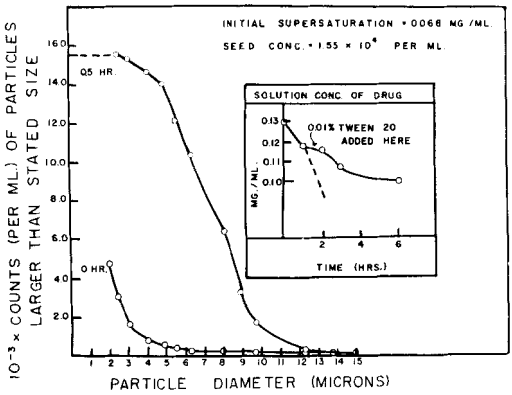


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THIS STUDY was undertaken to establish a technique whereby urine would be voided at a constant pH over a protracted time. Using this technique, the elimination of sulfaethidole (SETD) from human subjects was studied to determine the influence of controlled urinary pH on its excretion kinetics. That the elimination processes of some drugs are influenced by administration of agents which affect the acidity or alkalinity of the urine is well known. For example, Smith, Gleason, Stoll, and Ogorzalek (1) and others (2–6) have demonstrated that renal

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clearance of salicylates is increased when sodium bicarbonate or other alkalinizing agents are employed to increase pH of the urine. Similar results have been shown by Waddell and Butler (7) and by Mollaret, Rapin, Pocidalo, and Monsallier (8, 9) for phenobarbital, by Waddell and Butler (10) for 5,5-dimethyl-2,4-oxazolidinedione, by Gutman, *et al.* (11), for phenylbutazone analogs, and by Peterson, Finland, and Ballou (12) for sulfadiazine. It is generally accepted in clinical practice that the excretion process of chemotherapeutic sulfonamides can be hastened by administration of sodium bicarbonate. Also, it is well known that the elimination of many basic drugs is hastened by agents which acidify the urine (13–18).

The influence of urinary pH on the elimination of a drug such as SETD can be attributed to the pH dependence of renal tubular transfer of the drug. A large portion of SETD in the human body is contained in extracellular fluids, of which the blood is a large component. Blood passing through the nephron of the kidney is filtered of many of its components. The capillary networks of the glomeruli act as filter beds for the plasma, whereby most of the nonprotein substances filter passively through the glomeruli into the renal tubules. Many of the normal components of the glomerular filtrate are, in part, reabsorbed into the circulation from the tubules; absorption is effected by both active and passive processes, depending upon the substance in question. In addition, tubules may actively and passively secrete substances from the blood into the tubular fluid.

Drugs may be filtered through the glomeruli, secreted into the tubules, and reabsorbed from the tubules in ways similar to those which apply to normal blood components; however, knowledge of the active or passive nature of drug secretion and reabsorption at the renal tubule level is limited. For many drugs it is thought that passive diffusion may be more important than active transport in their excretion and reabsorption at the renal tubule level.

A difference in pH between plasma and tubular fluid can result in a considerable difference in the fraction of undissociated weakly acidic or weakly basic drug in these two fluids. In the absence of a specific transport mechanism it is usually the undissociated form of the drug which most readily diffuses between the two fluid compartments. The concentration gradient of the nonionized drug across the cellular barrier separating plasma from tubular fluid is, therefore, considered to be of primary importance in passive transport. Though it is difficult to determine the pH of fluid at sites in the tubule intermediate of the glomeruli and the bladder, the pH of the beginning and terminal processes can be readily determined by measuring plasma and urine pH. Plasma pH in humans is generally very constant, near 7.4, whereas urine pH may vary through a range of about 4.5 to 8.5 under different natural or experimental conditions.

Milne, Scribner, and Crawford (19) have shown that the pH of the tubular fluid and the pKa and partition coefficient of the drug would be expected to influence the renal tubular transfer of many weak acids and bases, just as such factors influence the absorption and secretion of drugs in the gastrointestinal tract (20–24). It was thought that by devising a technique for maintaining urine pH constant at several pH values, a convenient method would become available for assessing quantitatively, in kinetic terms, the effect of pH on the excretion of drugs. Since SETD is a weak acid which is eliminated virtually completely *via* the urinary route, and methodology for the kinetic elimination studies has been established (25–28), this appeared to be a suitable drug for such study.

EXPERIMENTAL

Human Studies.—Twelve healthy young adult white male human volunteers were employed in the excretion trials of this study. For most trials, food and fluid intake were not controlled.

Ammonium chloride and sodium bicarbonate were used, in the manner previously reported (29), to maintain the desired acidic or alkaline condition of the urine throughout the studies. Normally, a 3- to 4-Gm. dose of one of these agents was given in tablet form for 2 or 3 hours, until measurement of urine pH indicated that pH values of 5 or 8 had been attained. Smaller doses of these agents were administered, usually each hour, to maintain the pH at the desired value. The pH of the urine was monitored, usually every hour, to determine whether succeeding doses should be increased or decreased to maintain a constant pH.

After the desired urine pH was attained, a single 2-Gm. dose of powdered SETD suspended in water was administered orally. Samples of urine were collected, usually at hourly intervals, for at least 10 hours. Volumes of urine voided, pH, and free SETD excreted were measured in a total of 16 trials at a urinary pH near 8, and in 17 trials at a urinary pH near 5. All subjects were used in at least one trial at each of the two urinary pH values. One subject was studied to determine the effect of induced diuresis on the excretion profile. Another variation involved an excretion profile study with one subject where, in a single trial, the urine was maintained first at an acid pH and then at an alkaline pH.

Analytical Methods.—Urine pH measurements were made with a Beckman model G pH meter.

TABLE I.—ILLUSTRATION OF TYPICAL DOSAGE REGIMENS OF SODIUM BICARBONATE AND AMMONIUM CHLORIDE REQUIRED TO ATTAIN AND MAINTAIN CONSTANT URINARY pH IN HUMAN SUBJECTS

	Dosage of Ammonium Chloride, Gm.	Time, hr.	Urinary pH	Dosage of Sodium Bicarbonate, Gm.	Time, hr.	Urinary pH
Subject A	4.0	−1.25	...	4.2	−2.00	...
	...	−0.25	5.25	4.2	−1.00	...
	3.0	0	5.15	3.0	0	7.80
	2.0	1.17	5.07	1.2	1.00	8.10
	1.0	2.17	5.07	1.2	2.00	8.10
	1.0	3.33	5.03	1.2	3.00	7.95
	1.0	4.17	4.97	3.0	4.00	7.60
	1.0	5.00	5.00	3.0	5.00	7.90
	1.0	6.00	5.02	1.8	6.00	8.10
	1.0	7.00	5.03	1.8	7.00	8.10
	...	8.00	5.09	1.8	8.00	8.00
	...	9.33	4.87	1.8	9.00	8.10
	...	10.00	4.82	1.8	10.00	8.10
	...	12.00	4.82	...	11.00	8.00
Subject B	4.0	−1.00	...	4.2	−3.00	...
	3.0	0	...	4.2	−2.00	...
	1.0	1.00	5.30	4.2	−1.00	...
	1.0	2.00	5.30	4.2	0	7.50
	1.0	3.50	5.30	3.0	1.00	8.05
	1.0	4.00	5.30	3.0	2.00	8.00
	1.0	5.00	5.20	3.0	3.00	8.00
	1.0	6.00	5.10	1.2	4.00	8.10
	1.0	7.00	5.20	1.2	5.00	8.10
	1.0	8.00	5.30	1.2	6.00	8.00
	1.0	9.00	5.30	1.2	7.00	8.00
	...	10.00	5.30	1.8	8.00	8.00
	...	...	...	1.8	9.00	7.95
	...	...	...	...	9.50	7.85

Each urine sample, when collected, was rapidly brought to room temperature and the pH was determined immediately.

Urine samples were assayed for free SETD using a modification of the Frisk macro method (30). Spectrophotometric measurements of the final colored solutions, involving use of the Bratton-Marshall reagent (31), were made with a Beckman model B spectrophotometer, at a wavelength of 550 mμ.

RESULTS

Control of Urine pH.—Typical dosage regimens and the resulting urine pH are presented in Table I. Subjects were somewhat variable with regard to the quantities of ammonium chloride or sodium bicarbonate required to attain and maintain a constant pH; however, after gaining experience with this technique, and by monitoring the urine pH at about hourly intervals, it was a relatively easy matter to maintain urine pH within ±0.2 units over periods of at least 15 hours. The occasional incidence of nausea from the large doses of sodium bicarbonate and ammonium chloride was the principal difficulty encountered in employing this technique.

While good control of urine pH can be attained near pH 5 or pH 8, it was more difficult to maintain constant pH at intermediate values. It was concluded that this could not be accomplished conveniently to the degree of constancy desired for the excretion studies; therefore, studies on the SETD excretion profile were limited to values near pH 5 and pH 8.

Excretion Studies.—Typical data for the SETD excretion profile studies under conditions of constant pH are presented in Tables II and III. Since it has been shown previously (26, 27, 32) that oral doses of SETD are virtually completely absorbed, almost totally excreted in the urine, and that virtually all of the drug in the urine is measurable by use of the Bratton-Marshall reagent, interpretation of the excretion data was not difficult. As shown previously for this drug, rate of elimination can be expressed as

$$dW/dt = -k_b W \qquad \text{(Eq. 1)}$$

where W is the amount of drug remaining in the body at any time, t, or W = (dose − amount excreted), and k_b is the specific velocity constant for disappearance of drug from the body. Since

$$\log W = -(k_b/2.303)t + \text{constant} \qquad \text{(Eq. 2)}$$

k_b can be determined from the slope of the straight line resulting from a plot of log W *vs.* time (27). The biologic half-life, $t_{1/2}$, is determined for each trial from $t_{1/2} = 0.693/k_b$.

Plots of logarithm mg. of unexcreted drug *vs.* time, for single subjects, gave straight line plots after 1 or 2 hours following administration. Table IV illustrates the biologic half-life values obtained from inspection of the exponential plots of the urinary excretion data and the average urine pH as observed for each trial. Average acid urine pH values for subjects in 17 trials ranged from pH 4.5 to pH 5.3. When two trials are eliminated from the summary data, 15 trials fall within the pH range 4.8 to 5.2. The harmonic mean for $t^1/_2$ values observed in the 15 trials at pH 5 ± 0.2 is 11.4 hours with fiducial limits (26) of 9.9 to 13.5 hours. This corresponds to an arithmetic average k_b of 0.061 ± 0.004 hr.$^{-1}$, where ± designates standard error.

Average alkaline urine pH values for subjects in 13

TABLE II.—TYPICAL DATA FOR URINARY EXCRETION OF SETD,[a] URINE MAINTAINED AT pH 5

Dosage of Ammonium Chloride, Gm.	Hours	Urinary pH	Urine Volume, ml.	SETD per Sample, Mg.	Total Unexcreted SETD, mg.
4.0	−3.33	...	...	...	...
...	−1.83	5.3	...	...	...
1.0	0	...	...	...	...
1.0	1.33	5.1	248	22.3	1978
1.0	2.75	5.1	235	87.0	1891
1.0	3.75	5.1	114	124.3	1766
1.0	4.92	5.0	108	140.8	1626
...	6.08	5.0	119	132.1	1494
1.0	7.08	5.0	80	88.0	1406
1.0	8.08	5.1	100	90.0	1316
0.5	10.08	4.9	192	151.7	1164
...	11.08	4.8	149	82.0	1082
...	12.08	4.9	78	60.8	1021
...	13.23	5.0	57	51.3	970
0.5	14.08	5.0	50	37.0	933
...	15.08	4.9	75	45.6	887
...	17.75	...	391	104.0	783
...	23.00	...	946	200.6	582
...	25.59	...	489	86.1	497

[a] SETD, 2 Gm., administered orally at zero hour.

TABLE III.—TYPICAL DATA FOR URINARY EXCRETION OF SETD,[a] URINE MAINTAINED AT pH 8

Dosage of Sodium Bicarbonate, Gm.	Hours	Urinary pH	Urinary Volume, ml.	SETD per Sample, mg.	Total Unexcreted SETD, mg.
4.2	−2.75	...	...	...	...
4.2	−1.50	6.4	...	...	...
3.6	−0.25	8.1	...	...	...
0.6	0	8.6	...	...	...
3.6	1.00	8.1	70	294.0	1706
3.0	2.00	8.1	62	235.6	1470
3.6	3.08	8.1	95	247.0	1223
3.0	4.08	8.1	113	180.8	1043
3.0	5.08	8.1	99	142.6	900
2.4	6.08	8.1	86	123.9	776
2.4	7.66	8.1	130	179.4	597
...	8.75	8.0	100	108.0	489
3.0	9.25	8.0	44	44.0	445
...	10.59	8.1	130	75.4	369
...	11.59	8.0	105	39.9	329
...	13.59	8.0	130	85.8	244
...	14.83	7.6	158	63.2	180
...	19.75	...	580	118.9	62

[a] SETD, 2 Gm., administered orally at zero hour.

trials ranged from pH 7.4 to pH 8.1. When the three trials at the lowest pH are excluded, 10 trials are observed to fall within the average pH range of 7.9 to 8.1. The harmonic mean for $t_{1/2}$ values in the 10 trials at pH 8 ± 0.1 is 4.2 hours, with fiducial limits of 3.7 to 4.8. Average k_b is 0.165 ± 0.009 $hr.^{-1}$

Figure 1 is a presentation of the composite urinary excretion data from which Table IV values for $t_{1/2}$ were determined. It is a rather dramatic illustration of the adherence of the urinary excretion process, of many trials, to first-order kinetics in the time range studied. In addition, it gives a clear visual illustration of the dependence of the process upon the magnitude of the equilibrium urine pH. Figure 2 also illustrates this very well where an abrupt change in the slope of the excretion curve occurs when urine, being collected during a single trial, was suddenly changed from a pH of 5 to a pH of 8.

Though previous data had supported the opinion that, in the presence of adequate urine flow, elimination kinetics of SETD is relatively independent of urine volume (27), in the early phase of this study experimental verification of this point seemed desirable. During the course of an excretion study, a single subject who had consumed approximately 100 ml. of water each hour was suddenly changed to a regimen where he consumed almost 1000 ml. per hour for several hours. Data presented in Fig. 3 indicate that SETD excretion was essentially unchanged even by tenfold or greater increases in urine volume, so long as precautions were taken to maintain constant urine pH.

DISCUSSION

The fact that urine pH influences net elimination of SETD to the degree indicated in Table IV indicates that renal tubular transport of the drug plays an important role in the elimination process. Approximately 95% of the SETD in plasma is

TABLE IV.—BIOLOGIC HALF-LIFE FOR SETD IN HUMAN SUBJECTS AS A FUNCTION OF URINARY pH

Acid Range		Basic Range	
Av. Urine pH	$t_{1/2}$, hr.	Av. Urine pH	$t_{1/2}$, hr.
4.5	14.2	7.4	6.6
4.8	12.5	7.5	4.7
4.8	12.6	7.8	5.4
4.8	8.9	7.9	5.4
4.8	19.8	7.9	3.7
4.8	12.5	8.0	3.3
4.8	10.0	8.0	5.5
4.9	21.3	8.0	4.4
4.9	8.9	8.0	4.1
4.9	10.7	8.0	3.7
5.0	20.1	8.1	5.3
5.0	11.4	8.1	4.1
5.0	10.3	8.1	3.9
5.1	8.0	...	...
5.1	10.4	...	...
5.2	9.2	...	...
5.3	10.6	...	...
For 15 trials within pH range 4.8 to 5.2, Av. $t_{1/2}$, 11.4 hr.[a]		For 10 trials within pH range 7.9 to 8.1, Av. $t_{1/2}$, 4.2 hr.[a]	

[a] Harmonic mean $t_{1/2}$.

bound (33) and, therefore, only a small fraction of the total SETD is cleared in any single pass of fluids through the glomeruli. While the concentration of diffusible drug in the glomerular filtrate must be low, a condition favoring diffusion of SETD back into general circulation might be created through water reabsorption in the proximal tubule, thus increasing the concentration of drug in tubular fluid over that of unbound drug in the plasma. Furthermore, a decrease in pH of tubular fluid would favor reabsorption of drug by increasing the concentration of the more readily diffusible undissociated form of the drug.

The pKa of SETD, determined by potentiometric titration, is about 5.5, and the fraction of SETD which is undissociated is given by

$$(H^+)/[(H^+) + Ka]$$

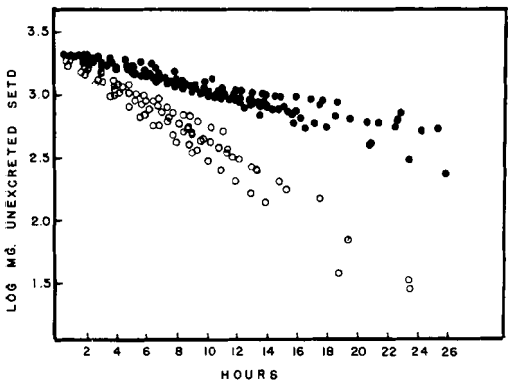


Fig. 1.—Exponential plot of the composite urinary excretion data for sulfaethidole following single 2-Gm. oral doses to 12 subjects in 30 trials. Darkened circles indicate data at constant urine pH near 5 and open circles indicate data where urine pH was maintained near 8 as described in the text.

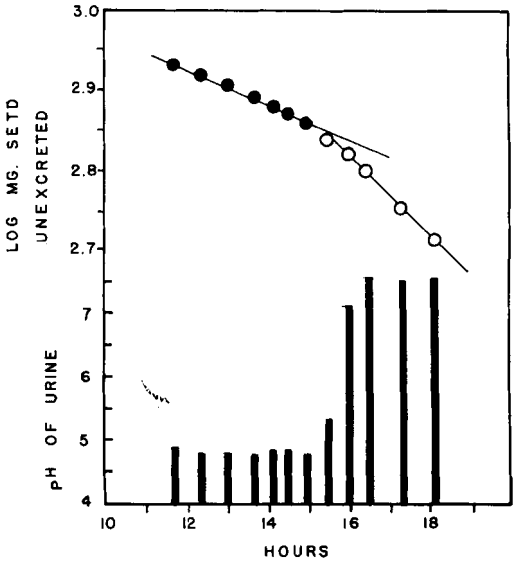


Fig. 2.—Exponential plot of SETD urinary excretion data for a single human subject after a 2-Gm. oral dose. The figure illustrates the abrupt change in slope of the exponential process when urine pH is changed from about pH 5 to pH 8.

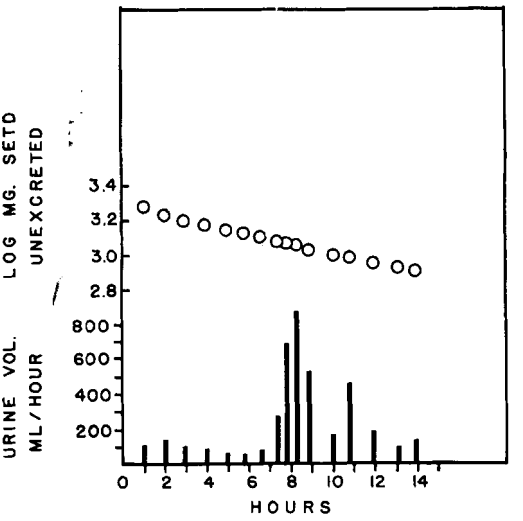


Fig. 3.—Exponential plot of SETD urinary excretion data for a single human subject after a 2-Gm. oral dose when urine pH was maintained near 5, but where the rate at which urine is voided was changed appreciably. The figure illustrates that the elimination rate is relatively independent of urine volume voided.

The fraction of SETD undissociated in plasma and urine under conditions of this study is as follows: plasma, pH 7.4, 1.2% free SETD is nonionized; urine, pH 8.0, 0.3% SETD is nonionized; urine, pH 5.0, 76% SETD is nonionized.

Although the pH of tubular fluid at sites for reabsorption of SETD is unknown, it seems fairly certain that under conditions of this study the fluid

would be alkaline when urine pH was maintained at pH 8 and acid when urine was maintained at pH 5. The longer biologic half-life for SETD under conditions of acid urine suggests that reabsorption of the drug from tubular fluids is favored by higher concentrations of undissociated drug. This concept is similar to those concerning gastrointestinal absorption which have seen considerable discussion in recent literature by Schanker, Brodie, Hogben, *et al.* (20–24). Milne, Scribner, Crawford (19), Gutman, *et al.* (11), and others have developed such thinking regarding factors influencing net drug elimination *via* the urinary route.

Though the pKa of SETD and other acidic and basic drugs apparently plays an important role in the mechanism of drug elimination, and though controlled urinary pH can exert a profound effect on the rate of net drug elimination, the intrinsic lipid solubility of the undissociated drug, concomitant administration of other drugs, and other factors must also be important in influencing the net elimination rate. It would seem important to obtain a clearer understanding of the mechanism of drug excretion by the kidney, and of the interrelationships of the physical-chemical properties of drugs and the mechanisms which come into play in body elimination of drugs. Such knowledge, coupled with additional knowledge of other body mechanisms for eliminating drugs and drug activity, has a bearing on the intelligent design of drugs and their dosage forms for controlling their duration of action.

The results of this study supplement and support, in a number of respects, previously reported findings concerning SETD excretion kinetics (25–28, 30, 32). In previous studies without benefit of controlled urinary pH, and using both blood concentrations and urinary excretion data, the $t_{1/2}$ for SETD in human subjects was shown to be about 8.4 hours (27). This value is about midway between the values of 4.2 and 11.4 hours, at controlled urine pH of 8 and 5, respectively, determined in this study. These results also give further reinforcement to the opinion that the $t_{1/2}$ values of drugs determined experimentally in human subjects are not to be construed as fixed values. Rather, they are subject to variation by a number of factors about which much additional data are necessary for a better understanding of elimination mechanisms and kinetics.

SUMMARY

1. A technique has been developed to permit control of the pH of urine being excreted by human subjects to ±0.1 pH unit, in the vicinity of pH 5 or pH 8, over periods of at least 15 hours.

2. Constant urinary pH in the vicinity of pH 5.0 can be attained by administering several grams of ammonium chloride each hour until the desired pH has been attained, and then increasing or decreasing the maintenance dose as indicated by hourly monitoring of urine pH. Constant urinary pH in the vicinity of pH 8.0 can be attained by employing sodium bicarbonate in a similar fashion.

3. Modification of urine pH in human subjects has a profound effect on the rate of elimination of sulfaethidole from the body.

4. The harmonic mean $t_{1/2}$ for SETD is 11.4 hours when urine pH is maintained at pH 5.0; the harmonic mean $t^{1}/_{2}$ is only 4.2 hours when urine pH is maintained at pH 8.0.

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Effects of Certain Compounds on Experimental Muscular Dystrophy

By NATHAN WATZMAN†, WILLIAM J. KINNARD, MARIO D. ACETO, and JOSEPH P. BUCKLEY

Several compounds which represent classifications of drugs known to have effects on muscular efficiency were administered to dystrophic animals in which atrophy was achieved utilizing a vitamin E-deficient diet. A myopathic mutation of the house mouse (dystrophia muscularis) was also utilized in certain portions of the study. Motor coordination, weight, muscle strength, and urinary creatine and creatinine were measured. The effects of some of the compounds on the isolated diaphragm muscle of normal and dystrophic mice were also studied. Norethandrolone, methimazole, and digoxin increased the ability of vitamin E-deficient rats to perform forced motor activity. The same compounds increased the offspring survival rate of vitamin E-deficient female rats. Potassium chloride (10 mg./Kg./day) temporarily increased the mean rotarod performance time and arrested loss of weight of dystrophic mice. Although there was no statistical difference between the life span of the control and drug-treated groups of strain-129 mice, the group receiving digoxin, 0.05 mg./Kg. subcutaneously, twice a week survived the longest.

MUSCULAR DYSTROPHY is a chronic disease manifested by a gradual weakening of the voluntary muscles. As the condition progresses, there occurs loss of function, mild contractions, and finally contractures in which the muscle is entirely inert and no longer capable of response to stimuli.

The etiology of muscular dystrophy has eluded investigators for many years, but it appears to be due to some defect in metabolism. The following metabolic alterations and abnormalities invariably appear in dystrophic muscle tissue: decreased concentration of creatine and increased concentration of creatinine in striated muscle (1); creatinuria (2); decreased concentration of extracellular potassium and magnesium (3); diminished glycogenolysis (4); increased collagen nitrogen (5); and decreased concentrations of the enzymes aldolase and phosphorylase (5).

In the light of these consistent findings, it seemed plausible to administer certain therapeutic agents which might metabolically or pharmacologically reverse the specific patterns elicited by the dystrophic conditions.

Methimazole (1-methyl-2-mercaptoimidazole),[1] one of the most active of the antithyroid agents (6), was selected as one of the test compounds. Increasing evidence exists that muscle weakness, tremors, and reduced muscle efficiency occur in hyperthyroidism. Du Toit (7) reported that the oxidative efficiency of phosphorylation is reduced in the presence of thyroxin, suggesting that muscle efficiency might be reduced in hyperthyroidism. Thomson and his associates (8) reported that hyperthyroidism was accompanied by tremors, atrophy, myasthenia, and muscle lesions. In their studies, denervation atrophy was accelerated by thyroxin and retarded by thiouracil, an antithyroid agent. In addition, Boldrini (9) using liver slices of hyperthyroid rats, showed that the creatine content of liver and muscle of hyperthyroid animals was less than that of normal animals. Creatine synthesis was also depressed and this was reversed by methylthiouracil, an antithyroid agent.

The wasting of muscle in muscular dystrophy suggested the use of norethandrolone (17 alpha-ethyl - 17 - hydroxy - 19 - nor - 4 - androsten - 3-one),[2] an androgenic hormone, which enhances protein anabolism by reversing negative nitrogen balance. Saunders and Drill (10), using the levator ani muscle of the rat reported that norethandrolone had marked myotrophic activity. The most familiar example of the anabolic effects of the androgens is the rapid growth and muscular development of the adolescent male. Methandrostenolone,[3] another androgenic steroid with anabolic properties, was also selected as a test compound.

Digoxin[4] is widely used in the treatment of congestive heart failure. It acts directly on the myocardium to increase muscle tone and contractile force, thereby increasing the mechanical efficiency of the failing heart (11). This compound is deposited in skeletal muscle as well as the myocardium and might possibly produce an effect in skeletal muscle similar to that produced in the failing heart.

The finding of increased collagen nitrogen (12)

Received March 26, 1962, from the Pharmacology Laboratories, University of Pittsburgh, School of Pharmacy, Pittsburgh, and Northeast Louisiana State College, School of Pharmacy, Monroe.
Accepted for publication May 11, 1962.
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Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
[1] Marketed as Tapazole by Eli Lilly & Co.
[2] Marketed as Nilevar by G. D. Searle & Co.
[3] Marketed as Dianabol by Ciba Pharmaceutical Co.
[4] Marketed as Lanoxin by Burroughs Wellcome & Co.

in the dystrophic muscle suggests an increase in fibrous tissue resulting from an inflammatory condition. This fact, coupled with evidence of diminished glycogenolysis (4) led to the consideration of an anti-inflammatory corticosteroid as one of the experimental compounds. Methylprednisolone acetate,[5] the 6-methyl derivative of prednisolone (delta-1-hydrocortisone), was selected because it has been reported to be more potent than prednisolone and less prone to produce early side effects (13).

Evidence of electrolytic disturbances in atrophic muscle such as decreased concentrations of extracellular potassium and magnesium ions (14) and increased sodium and calcium ions (14) led to the selection of the electrolytic compound, potassium chloride, as another test substance. Dystrophic muscles have been reported to have a slightly lower potassium content than normal muscle (15). Overholt and his associates (16) have reported the prevention of periodic paralysis associated with hyperthyroidism by the use of a high potassium diet.

Finally, three other pharmacologically active agents were utilized on modified *in vitro* diaphragm preparations. Ephedrine was employed because of evidence that this substance reversed the effects of fatigue, a condition not unlike that of muscular dystrophy. Burns (17) demonstrated in dogs that ephedrine causes a marked and prolonged rise in tension of skeletal muscle fatigued by repeated stimulation of the sciatic nerve. The second compound selected for *in vitro* study was ouabain (g-strophanthin), a cardiac glycoside having a very quick onset of action. Cattell (18) has shown that this compound produces an increase in force of striated muscle contraction. Caffeine is known to increase the amplitude of muscle contractions (19). Contractions of isolated nerve-muscle preparations are enhanced and muscle made less susceptible to fatigue if low concentrations of xanthines are present in the bath solution (20); therefore, theophylline (21) was selected as the third compound.

Several experimental methods for the development of dystrophic symptoms in laboratory animals are available to investigators in this field. Muscular weakness and wasting similar to human muscular dystrophy have been accomplished by vitamin E-deficient (22) or choline-deficient (23) diets in many species of animals. Also, through the efforts of the staff at Jackson Memorial Laboratories, Bar Harbor, Maine, a myopathic mutation appeared among a colony of inbred strain-129 mice (dystrophia muscularis) in 1951. The clinical manifestations of the disease in this species are first recognizable at $3^1/_2$ weeks when ataxia and paresis of the hind limbs are observed.

The objectives of this study were: (*a*) to determine if the test compounds delay or arrest, to any degree, the onset of experimental muscular dystrophy and (*b*) to determine if the test compounds affect the contraction of normal and fatigued muscle.

EXPERIMENTAL

Development of Experimental Muscular Dystrophy.—Muscular weakness and ataxia were observed in albino Wistar rats after 5 months feeding on the vitamin E-deficient diet utilized successfully by Olcott (22).

Dose Regimen.—Thirty albino Wistar rats, weighing approximately 100 Gm., were divided into five groups of six animals each (two males and four females). Group 1 received a normal rat diet (Purina Rat Chow); group 2 received a vitamin E-deficient diet; group 3 received the vitamin E-deficient diet and norethandrolone, 2 mg./Kg. orally; group 4 received the vitamin E-deficient diet and digoxin, 0.2 mg./Kg. initially and then 0.1 mg./Kg. daily by oral route; group 5 received the vitamin E-deficient diet and methimazole, 4 mg./Kg. orally. Drugs were administered daily, 6 days a week.

One hundred and sixty-four pairs of dystrophic mice (dystrophia muscularis, Jackson Memorial Laboratories, Bar Harbor, Maine) and their littermate controls were divided into 14 groups of not less than nine pairs each. Twenty pairs comprised the control group. Group 1 (controls) received no drugs; group 2 received norethandrolone, 2 mg./Kg. orally; group 3 received norethandrolone, 10 mg./Kg. orally; group 4 received methimazole, 4 mg./Kg. orally; group 5 received methimazole, 20 mg./Kg. orally; group 6 received digoxin, 0.2 mg./Kg. for 1 week and then 0.05 mg./Kg. daily by the subcutaneous route; group 7 received digoxin, 0.2 mg./Kg. initially for one dose and then 0.1 mg./Kg. daily by the subcutaneous route; group 8 received digoxin, 0.2 mg./Kg. daily by the subcutaneous route; group 9 received potassium chloride, 10 mg./Kg. orally; group 10 received potassium chloride, 50 mg./Kg. orally; group 11 received potassium chloride, 100 mg./Kg. orally; group 12 received methylprednisolone acetate, 10 mg./Kg. intramuscularly; group 13 received methylprednisolone acetate, 100 mg./Kg. intramuscularly; and group 14 received methandostrenolone, 10 mg./Kg. orally. Drugs were administered daily, 7 days per week except methylprednisolone acetate which was given twice weekly. The animals were weighed every fourth day and careful records maintained on their life spans.

Measurement of Motor Coordination.—Rats were placed on an electrically driven rotarod (24), $1^1/_4$ inches in diameter, which was set to turn at 18 r.p.m. The animals were forced to walk the turning rod without side excursions until they fell off. The rats were trained for a period of 1 week and then the relative forced motor activity and muscle coordination were determined by the length of time

[5] Marketed as Depo-Medrol by The Upjohn Co.

the animals could maintain their position on the rod, allowing a maximum of 2 minutes per animal. Coordination in strain-129 mice (dystrophia muscularis) was measured in the same manner as the rats, using a rod of smaller diameter (1 inch), set to rotate 11 r.p.m.

Measurement of Muscle Strength.—This factor was measured in rat offspring and dystrophic mice by placing them on a vertical screen (90 degrees) and recording the length of time they could remain on it. The maximum experimental time was established at 60 seconds and the average of each group recorded in seconds.

Measurement of Urinary Creatine-Creatinine Ratio.—Twenty-four hour urine samples were obtained from rats placed in individual metabolism cages every 2 weeks. A urine analysis of creatine and creatinine was determined according to a modification of Folin's method (25).

Survival of Offspring of Pretreated Vitamin E-Deficient Female Rats.—Female rats of all groups were mated with normal males 90 days following the initiation of the vitamin E-deficient diet. On the first day of mating, a single oral dose of 0.5 Gm. of wheat germ oil (not less than 2 i.u. vitamin E per Gm.) was administered to each female to assure proper reproductive ability. Size of litters and survival rates as well as motor coordination and muscle force of the offspring were recorded.

In Vitro Studies.—The effect of the test compounds on the amplitude of contractions of the diaphragm muscle of normal and dystrophic mice (dystrophia muscularis) was studied utilizing a modified method of Bülbring (26). The test compounds were added to the muscle bath to produce the following concentrations: potassium chloride (1:1500); ephedrine sulfate (1:1000); ouabain (1:35,000,000); theophylline ethylenediamine (1:50,000). A similar study was done, utilizing the fatigued diaphragm of normal and dystrophic mice. A submaximal threshold was established for each preparation and then the muscle was fatigued by stimulating every 10 seconds until a 50% decrease in amplitude of contraction was achieved. The test compounds were then administered directly into the bath and effects on the amplitude of contractions and recovery recorded.

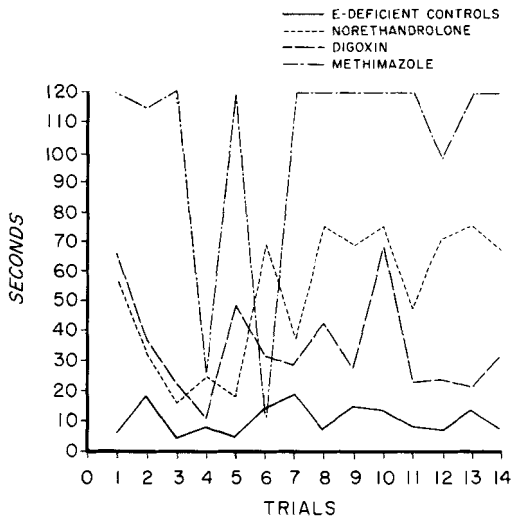


Fig. 1.—Effects of norethandrolone, digoxin, and methimazole on he rotarod performance times of vitamin E-deficient male rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

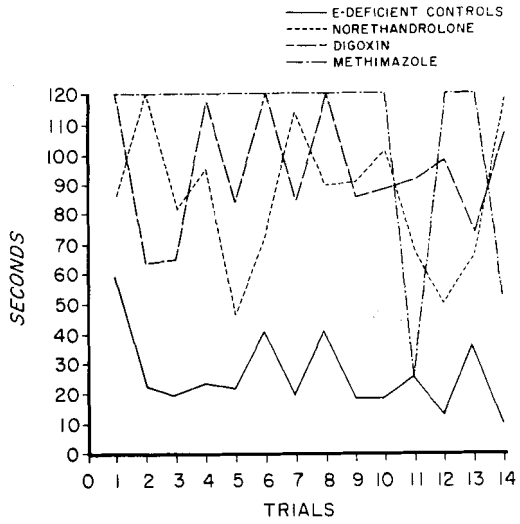


Fig. 2.—Effects of norethandrolone, digoxin, and methimazole on the rotarod performance times of vitamin E-deficient female rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

RESULTS AND DISCUSSION

The initial pilot study of this investigation in experimental muscular dystrophy utilized albino rats of both sexes. After 5 months on a vitamin E-deficient diet it was quite apparent that there was a significant difference in the abilities of the treated groups to perform on the rotarod apparatus (Figs. 1 and 2). Methimazole-treated rats demonstrated the greatest ability to perform forced motor activity. The data further indicated that the methimazole, digoxin, and norethandrolone-treated rats demonstrated greater ability to perform forced motor activity than the untreated vitamin E-deficient rats. This was true of both sexes (Figs. 1 and 2), and it appears that the compounds in some way alleviated the paralysis-producing phenomenon of a vitamin E-deficient diet.

The consistently lower weights of the methimazole-treated animals from the fifth week on (Fig. 3) are unexplainable in view of the fact that since methimazole is an antithyroid compound, it would be expected that animals treated with this compound might be heavier than the nontreated rats.

The data on the urinary creatine-creatinine ratios were inconclusive. There appeared to be no correlation between loss of motor ability and creatine-creatinine values. The vitamin E-deficient control ratios were moderately higher than the other groups but were generally within the normal 0.1–1.0 range.

The moderate success of the experimental compounds in the pilot study on vitamin E-deficient rats gave impetus to the investigation of the offspring of vitamin E-deficient females. As observed by Olcott (12), offspring of vitamin E-

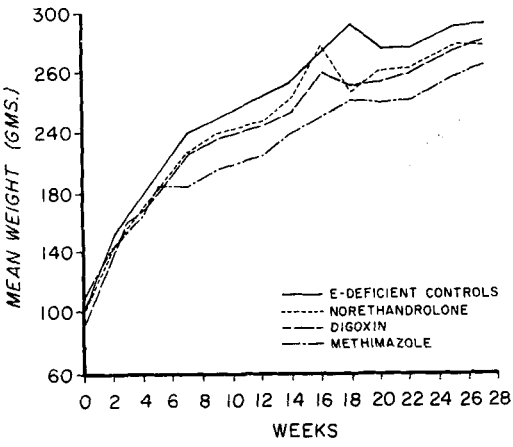


Fig. 3.—Effects of norethandrolone, digoxin, and methimazole on the weights of vitamin E-deficient female rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

deficient female rats developed a temporary muscular weakness on about the twentieth day of life. The phenomenon of muscular involvement in vitamin E-deficient animals is not understood. It would appear that the characteristic symptoms are genetically transferred to offspring. However, the temporary nature of these abnormalities at a specific age is not consistent with the fixed, permanent nature of genetics. This investigation corroborated Olcott's results and, in addition, elaborated two significant findings. A total of 74 offspring were produced by all groups (Table I). The normal control group

TABLE I.—EFFECTS OF NORETHANDROLONE, METHIMAZOLE, AND DIGOXIN ON OFFSPRING SURVIVAL OF VITAMIN E-DEFICIENT ALBINO RATS

	Group[a]				
	1	2	3	4	5
Offspring, No.	27.0	14.0	15.0	12.0	6.0
Offspring litter	9.0	5.0	5.0	4.0	3.0
Survival, %	70.3	0.0	26.6	25.0	50.0

[a] Group 1, normal control; group 2, E-deficient control; group 3, norethandrolone; group 4, digoxin; and group 5, methimazole.

had a mean of nine offspring per litter whereas the other groups ranged between three and five animals per litter. Nineteen offspring from normal control females survived, three from methimazole-treated females, four from norethandrolone-treated females, and three from digoxin-treated females. Per cent survival was fairly good for all groups with the exception of the vitamin E-deficient control group of which all offspring died within 18 days. The normal control group had a significantly higher per cent survival, 70.3%. The evidence thus indicated that all of the test compounds administered to the females increased the ability of the offspring of vitamin E-deficient rats to survive. However, in the light of the role of vitamin E as an antioxidant, the mechanisms by which the test compounds were able to increase offspring survival rate could not be resolved.

The second notable finding was that the offspring of norethandrolone-treated females were able to perform forced motor activity consistently better than the offspring of normal females (Fig. 4) and they experienced only slight difficulty in remaining on the vertical screen. In general, there appeared to be no correlation between motor coordination (as measured on the rotarod) and muscle strength (as measured by the vertical screen). This was evidenced by the poor performance of the digoxin offspring on the rotarod (Fig. 4), and their excellent agility on the vertical screen. Although norethandrolone produced highly beneficial effects in the vitamin E-deficient dystrophic rats, the effects of this compound in human muscular dystrophy has been discouraging. Dowben and Perlstein (27) have reported that the administration of 0.5 mg./Kg. of norethandrolone daily failed to produce an improvement in muscle function and strength.

The effects of the experimental compounds on the life span, motor coordination, and muscle strength of the strain-129 dystrophic mouse were observed. Rotarod performance graphs, life-span charts, cage score and weight graphs were carefully scrutinized to determine if any of the test compounds were successful in simultaneously (*a*) prolonging the life-span, (*b*) increasing motor performance times, (*c*) increasing the vertical cage scores, (*d*) increasing weight or at least arresting loss of weight. The data indicate that potassium chloride in the dose 10 mg./Kg. (group 9) was able to produce these responses temporarily (Figs. 5 and 6). From the 16th–64th day of therapy, mean rotarod performance times consistently ranged from 10.0–39.6 seconds. Thereafter the scores steadily decreased to zero. In comparison, the control dystrophics (group 1) never achieved a mean score higher than 5.4 seconds. Secondly, the mean weights of the potassium chloride-treated (10 mg./Kg.) animals ranged consistently between 13–17 Gm. with no apparent pattern of steady weight loss through the 120th day of drug administration. The mean cage

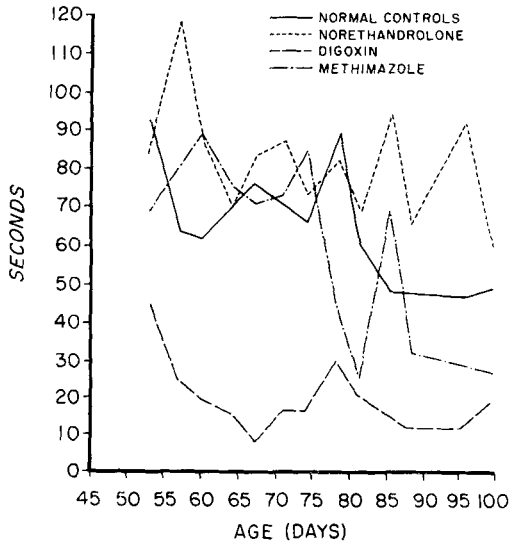


Fig. 4.—Effects of norethandrolone, digoxin, and methimazole on the rotarod performance times of the offspring of vitamin E-deficient female rats.

scores ranged steadily from 25.0–53.0 seconds through the 104th day of therapy whereas the control scores ranged from 7.0–28.0 seconds. Furthermore, the mean life-span of the potassium chloride-treated group was prolonged 16 days over that of the controls (Table II). However, statistical analysis showed this to be an insignificant response. The temporary beneficial results of potassium chloride in this study were understandable in the light of potassium evoked muscular improvements noted in the literature and previously discussed in the statement of the problem. Although norethandrolone has been reported to increase the survival time of strain-129 dystrophic mice (28), daily administration of this compound failed to increase the life-span of this strain of mice in this present study.

Although there was no statistical difference between the life spans of the untreated and drug-treated groups of strain-129 dystrophic mice, the group receiving digoxin 0.05 mg./Kg. subcutaneously twice a week (group 6) survived the longest. There appeared to be no correlation between mean rotarod performance times, mean cage scores, and life spans.

The results of the *in vitro* studies indicated that ouabain (1:35,000,000) and potassium chloride (1:1500) increased the amplitude of the contractions of the diaphragm muscle obtained from normal mice, 20.4 and 11.5%, respectively. None of the test compounds was able to evoke a change in the amplitude of diaphragm contractions of the dystrophic mouse. However, when the diaphragm muscle was partially fatigued, potassium chloride (1:1500) and ephedrine sulfate (1:1000) produced significant mean per cent recoveries, 33.5 and 20.3, respectively. Potassium chloride also produced a mean per cent recovery of 20.0 in the fatigued diaphragm of the normal mouse. The

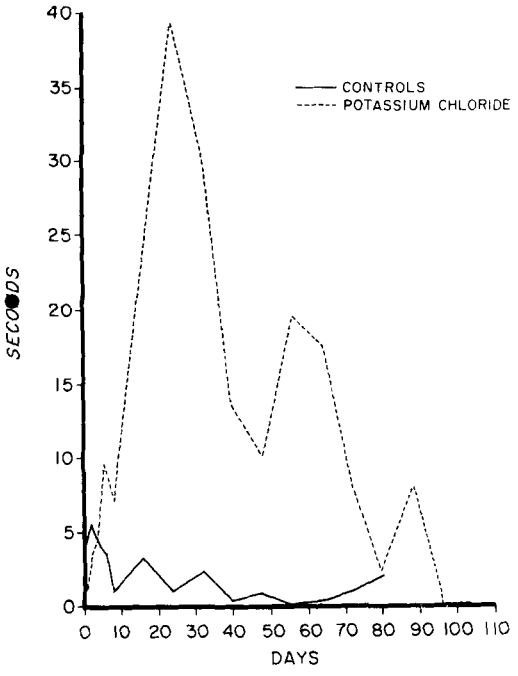


Fig. 5.—Effects of 10 mg./Kg. daily of potassium chloride on the rotarod performance times of dystrophic mice (dystrophia muscularis).

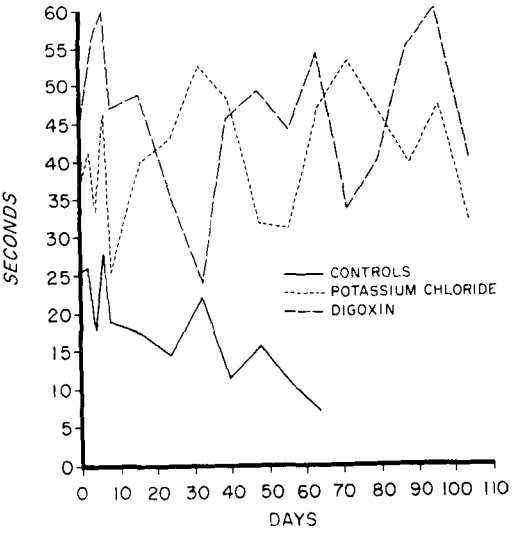


Fig. 6.—The effects of 10 mg./Kg. daily of potassium chloride and 0.05 mg./Kg. daily of digoxin on the vertical cage scores of dystrophic mice (dystrophia muscularis).

TABLE II.—EFFECTS OF EXPERIMENTAL COMPOUNDS ON LIFE SPAN OF STRAIN-129 DYSTROPHIC MOUSE (DYSTROPHIA MUSCULARIS)

Drug	Dose, mg./Kg.	Route of Administration	No. Animals	Mean Life Span ± S.D., Days
Controls	...	...	20	123 ± 20
Digoxin[a]	0.05	subcu.	9	151 ± 49
Digoxin[a]	0.10	subcu.	10	132 ± 50
Digoxin	0.20	subcu.	13	122 ± 31
Methimazole	4.00	oral	9	109 ± 34
Methimazole	20.00	oral	11	95 ± 17
Potassium chloride	10.00	oral	10	139 ± 48
Potassium chloride	50.00	oral	9	127 ± 41
Potassium chloride	100.00	oral	10	104 ± 26
Norethandrolone	2.00	oral	9	99 ± 23
Norethandrolone	10.00	oral	9	100 ± 14
Methandrostenolone	10.00	oral	16	100 ± 15
Methylprednisolone[b]	10.00	i.m.	10	130 ± 45
Methylprednisolone[b]	100.00	i.m.	18	92 ± 6

[a] 0.2 mg./Kg. initially (subcutaneously). [b] Administered twice weekly.

moderate success of potassium chloride in both *in vitro* and *in vivo* experiments on this special strain of mouse indicates that further investigation into the role of potassium as well as other ions should be carried out.

CONCLUSIONS

1. Methimazole-, digoxin-, and norethandrolone-treated rats of both sexes fed on a vitamin E-deficient diet demonstrated greater ability to perform forced motor activity than the untreated control groups.

2. Norethandrolone, digoxin, and methimazole increased the offspring survival rate of vitamin E-deficient female rats.

3. There appeared to be no correlation between motor coordination and muscle strength in the offspring of vitamin E-deficient female rats.

4. Oral dose of potassium chloride, 10 mg./Kg., appears to temporarily improve motor coordination in the strain-129 dystrophic mouse (dystrophia muscularis).

5. There appears to be no correlation between weights, motor coordination, and muscle strength in the strain-129 dystrophic mouse (dystrophia muscularis).

6. There was no statistical difference between the life spans of the control group and the drug-treated groups of strain-129 dystrophic mice (dystrophia muscularis).

7. Potassium chloride (1:1500) and ephedrine sulfate (1:1000) increased the amplitude of contractions in the partially fatigued diaphragm muscle of the strain-129 dystrophic mouse (dystrophia muscularis).

8. None of the test compounds appears to significantly delay or arrest the onset of experimental muscular dystrophy.

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Gas Chromatography of Alkaloids, Alkaloidal Salts, and Derivatives

E. BROCHMANN-HANSSEN and A. BAERHEIM SVENDSEN

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tracts of alkaloidal crude drugs and to direct gas chromatography of alkaloidal salts without prior isolation of the base.

EXPERIMENTAL

A Barber-Colman model 15 gas chromatograph equipped with an argon β-ionization detector was used for the experimental work. The columns were glass U-tubes 4 to 8 feet in length and having an inner diameter of 3 mm. The solid support was Gas-Chrom P, 100–140 mesh, washed with concentrated hydrochloric acid and methanolic potassium hydroxide and treated with hexamethyldisilazane (5). The siliconized material was immediately coated with 0.1% polyethylene glycol 9000 and, after drying, with about 1% silicone rubber SE-30. Both stationary phases were applied by means of solutions in toluene as described by Horning, *et al.* (6). The treatment with polyethylene glycol was found to reduce the adsorptive effects of the support material to an appreciable extent (5). The alkaloids were introduced with a Hamilton microliter syringe as 1.0 μl. of a 0.5 to 1.0% solution in tetrahydrofuran, acetone, or chloroform. Addition of methanol was sometimes used to dissolve alkaloidal salts.

Most extracts of crude drugs were prepared by conventional methods and purified by immiscible solvent extraction. Opium, however, was extracted by treatment with a cationic exchange resin (7).

RESULTS AND DISCUSSION

The retention times of alkaloidal bases, salts, and derivatives are listed in Table I.

Alkaloidal Salts.—The alkaloids could be gas chromatographed directly as salts without prior liberation of the base. This was especially true if the acid component was an organic acid or a hydrogen halide. However, even sulfates, phosphates, and nitrates could be gas chromatographed directly. The salts dissociated in the flash heater, which was maintained at about 325°, and the alkaloids were eluted as the free bases. Therefore, no difference

TABLE I.—RETENTION TIMES IN MINUTES OF ALKALOIDS AND ALKALOIDAL DERIVATIVES

Compound	Column Temp. 175°[a]	200°[b]	225°[c]
Apoatropine	5.2	...	...
Apomorphine	...	13.0	4.6
Apomorphine hydrochloride	...	...	4.6
Atropine	8.3	...	...
Atropine sulfate	5.2 8.3	...	...
Brucine	...	...	36.8
Brucine sulfate	...	...	36.8
Caffeine	2.4	...	...
Caffeine citrated	2.4	...	...
Caffeine hydrobromide	2.4	...	...
Caffeine hydrochloride	2.4	...	...
Cinchonidine	...	8.4	3.6
Cinchonine	...	8.2	3.5
Cinchonine sulfate	...	...	3.5
Cocaine	8.6	2.6	...
Cocaine hydrochloride	8.6	2.6	...
Codeine	16.4	4.2	1.7
Codeine hydrochloride	...	...	1.7
Codeine phosphate	...	...	1.7
Cotarnine	8.0[d]	...	...
Cryptopine	...	26.0	8.6
Cryptopine oxalate	...	...	8.6
Diacetylmorphine	40.2	8.7	3.5
Diacetylmorphine hydrochloride	...	...	3.5
Dihydrocodeinone	18.3	5.0	2.3
Dihydrocodeinone bitartrate	...	...	2.3
Dihydromorphine	23.4	5.6	2.4
Dihydromorphinone	27.5	6.2	2.9
Dihydromorphinone hydrochloride	...	...	2.9
Emetine	...	...	75.0
Ethylmorphine	17.7	4.7	2.2
Ethylmorphine hydrochloride	...	...	2.2
Eucaine	2.1	...	...
Eucaine hydrochloride	2.1	...	...
Gelseminine	...	...	4.6
Gelseminine sulfate	...	...	4.6
Homatropine	5.0	...	...
Homatropine hydrobromide	5.0	...	...
Hydrastine	...	...	10.1
Hydrastine hydrochloride	...	...	10.1
Hydrastinine	3.7[d]	...	...
Hydrastinine hydrochloride	3.7[d]	...	...
Laudanosine	...	10.0	3.8
3,O-Monoacetylmorphine	24.0	6.0	2.7
6,O-Monoacetylmorphine	29.6	6.8	2.9
Morphine	27.2	6.0	2.8
Morphine sulfate	...	...	2.8
Nalorphine	41.0	8.9	3.6
Noscapine	...	45.6	13.7
Neopine	17.5	4.4	2.0
Neopine hydrobromide	...	...	2.0
Papaverine	...	17.4	6.1
Papaverine hydrochloride	...	...	6.1
Physostigmine	8.8[d]	...	...
Physostigmine salicylate	8.8[d]	...	...
Pilocarpine	6.5	...	...
Pilocarpine hydrochloride	6.5	...	...
Quinidine	...	...	5.8
Quinidine sulfate	...	...	5.8
Quinine ethylcarbonate	...	19.1	6.5
Quinine	...	15.7	5.8
Quinine hydrochloride	...	...	5.8
Scopolamine	13.7	...	...
Scopolamine hydrobromide	13.7	...	...
Sparteine	2.1	...	...
Sparteine sulfate	2.1	...	...
Strychnine	...	34.0	11.7
Strychnine nitrate	...	...	11.7
Thebaine	23.6	5.7	2.5

Column, 6 ft. long, 3 mm. I.D.; packing, 1.15% silicone rubber SE-30 on Gas-Chrom P, 100–140 mesh. [a] Inlet pressure, 20 lbs. p.s.i., flow rate, 25 ml./min. [b] 22 lbs., 25 ml./min. [c] 24 lbs., 25 ml./min. [d] Major peak; decomposition resulted in additional minor peaks.

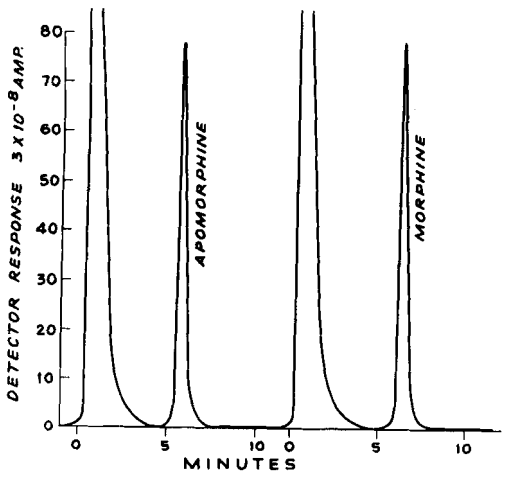


Fig. 1.—Gas chromatograms of apomorphine and morphine as the trimethylsilyl ethers. Column, 4 feet long and 3 mm. I.D.; packing, 1.2% silicone rubber SE-30 on Gas-Chrom P, 100–140 mesh; temp., 200°; inlet pressure, 20 lbs. p.s.i.

in retention time could be observed whether the alkaloid was applied as the salt or the base, Table I. Atropine sulfate produced two peaks, one of which appeared to be apoatropine resulting from dehydration in the flash heater. Although no difficulties were encountered during this work, the liberated mineral acids may in the long run have an adverse effect on the column and/or the detector.

Phenolic Alkaloids.—Phenolic alkaloids are often difficult to gas chromatograph because of adsorptive effects. Morphine generally gave only about one-half the peak height which one would expect on the basis of the amount applied. Apomorphine with two phenolic hydroxyl groups also produced a badly tailing peak. Such peaks are unsuitable for quantitative work. This difficulty was overcome by converting the phenols to the trimethylsilyl ethers (8). To a solution of 50 mg. of alkaloid in 3 ml. of tetrahydrofuran was added 2 ml. of hexamethyldisilazane. The mixture was allowed to stand in a well-closed container for about 24 hours and then gas chromatographed directly without isolation of the reaction product. Gas chromatograms of the trimethylsilyl ethers of morphine and apomorphine are illustrated in Fig. 1.

Extracts of Crude Drugs.—Figures 2 to 5 represent gas chromatograms of alkaloids extracted from several crude drugs. Where the alkaloidal mixture spans a wide range of vapor pressures, temperature programing is often useful to speed up the elution and obtain the high boiling components as relatively sharp peaks, Fig. 5. The gas chromatographic method may be used for identification of crude drugs, alkaloidal extracts and tinctures, and to differentiate between varieties of crude drugs having somewhat different alkaloidal composition. It also lends itself to a study of the various factors which may be expected to influence alkaloid production in plants.

Decompositions.—Some alkaloids could not be gas chromatographed without decomposition. Physostigmine, for example, gave two to four peaks, depending on the temperature of the flash heater. All attempts to produce a single-peak gas chromatogram of physostigmine were in vain. Hydrastinine and cotarnine also gave complex chromatograms containing several peaks. Atropine and scopolamine, which normally produced sharp, single-peak chromatograms, would under certain

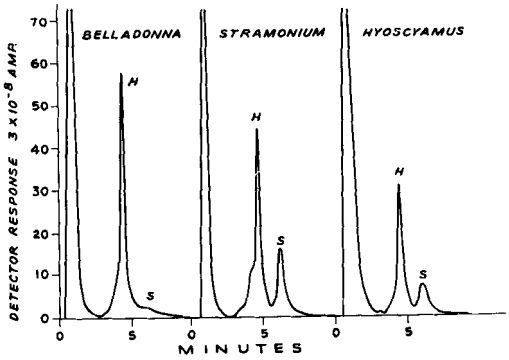


Fig. 2.—Gas chromatograms of extracts of the leaves of belladonna, stramonium, and hyoscyamus on silicone rubber SE-30. H, hyoscyamine; S, scopolamine. Column, 6 feet long and 3 mm. I.D.; temp. 200°.

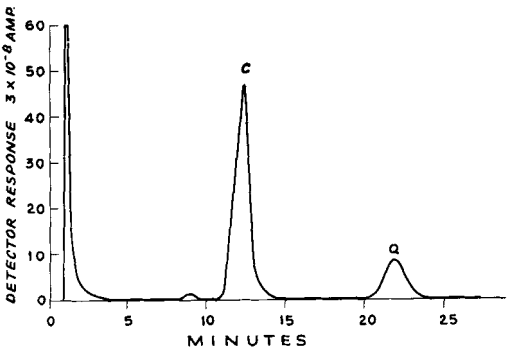


Fig. 3.—Gas chromatogram of an extract of cinchona bark on silicone rubber SE-30, 1.15%. C, cinchonine + cinchonidine; Q, quinine + quinidine. Column, 8 feet long and 3 mm. I.D.; temp. 200°.

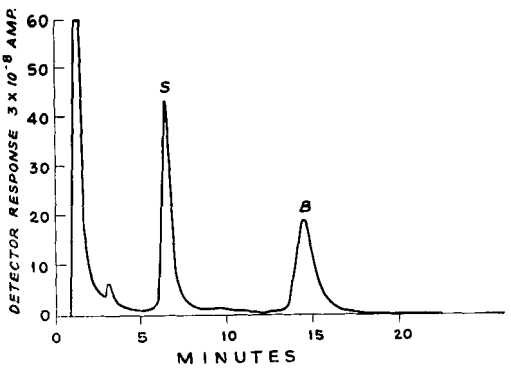


Fig. 4.—Gas chromatogram of an extract of nux vomica on silicone rubber SE-30, 1.15%. S, strychnine; B, brucine. Column, 8 feet long and 3 mm. I.D.; temp. 245°.

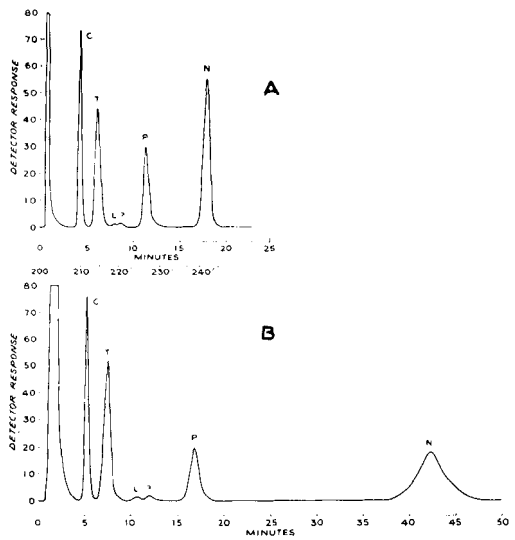


Fig. 5.—Gas chromatograms of the non-phenolic alkaloids of opium on silicone rubber SE-30, 1.15%. A, temperature programing; B, isothermal at 213°. C, codeine; T, thebaine; L, laudanosine; P, papaverine; N, noscapine. Column, 8 feet long and 3 mm. I.D.

operating conditions give two or even three peaks each. One of these appeared to represent the dehydrated alkaloid because the retention value of one of the peaks from atropine was identical with that given by apoatropine prepared according to Hesse (9). The degree of dehydration was found to be associated with the amount of glass wool placed on top of the column packing, and with the temperature of the flash heater. When a fairly large amount of glass wool was used, the degree of decomposition decreased as the flash heater temperature was reduced, but could never be entirely eliminated. When the amount of glass wool was reduced to a very small amount or removed completely, no decomposition took place even at flash heater temperatures of 350°. Similar dehydration of certain steroids has been described as a function of the flash heater temperature by Horning, *et al.* (10).

Hydrolysis and transesterification reactions were also sometimes noticed. Diacetylmorphine was eluted as a sharp peak when chromatographed alone. In mixtures with codeine, morphine or other alcoholic or phenolic substances, reactions taking place in the flash heater gave rise to several new esters not present in the original solution. Both 3, O-monoacetylmorphine (11) and 6, O-monoacetylmorphine[1] gave single peak chromatograms in the absence of glass wool. An apparent catalytic effect of glass wool resulted in peaks corresponding to morphine, monoacetyl- and diacetylmorphine.

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[1] We are indebted to Dr. E. L. Way for supplying us with a sample of this material.

Antiradiation Compounds III

N-2-Mercaptoethylpiperazines

By WILLIAM O. FOYE and DOUGLAS H. KAY

For the purpose of obtaining 2-mercaptoethylamine derivatives having an additional basic function located two carbons distant from the nitrogen, several N-2-mercaptoethylpiperazines have been synthesized. Procedures were employed that involved conversion of the 2-hydroxyethylpiperazines to the corresponding bromo compounds with subsequent thiolation, as well as those employing ethylene sulfide. Both 1-(2′-mercaptoethyl)piperazine and 1,4-bis(2′-mercaptoethyl)piperazine were found to give some protection to mice exposed to 575 roentgens of X-irradiation.

MERCAPTANS have been recognized as effective protective agents against the lethal effects of ionizing radiation, but all of the significantly successful compounds of this variety have so far had a basic function located two or three carbons distant from the sulfhydryl group. The most outstanding examples of the mercaptoamines are 2-mercaptoethylamine (1) and 2-mercaptoethylguanidine (2). Some question remains regarding the basicity of 2-acylthioethylamines, however, which were found to have appreciable antiradiation effects in mice (3), since the acyl groups shift to the nitrogen in neutral or alkaline media (3, 4). Otherwise, a fairly strongly basic group has been required in mercaptans for antiradiation properties, so it appeared desirable to prepare mercaptoamines having an additional basic group located two carbons from the parent amine.

Received March 27, 1962, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.
Accepted for publication May 31, 1962.
This project was carried out under a contract (DA-49-193-MD-2029) with the Office of the Surgeon General, U. S. Army Medical Research and Development Command.
The authors express their gratitude for this support as well as for the testing data supplied by Dr. D. P. Jacobus.
Presented to the Scientific Section, A.Ph.A., Las Vegas meeting, March 1962.

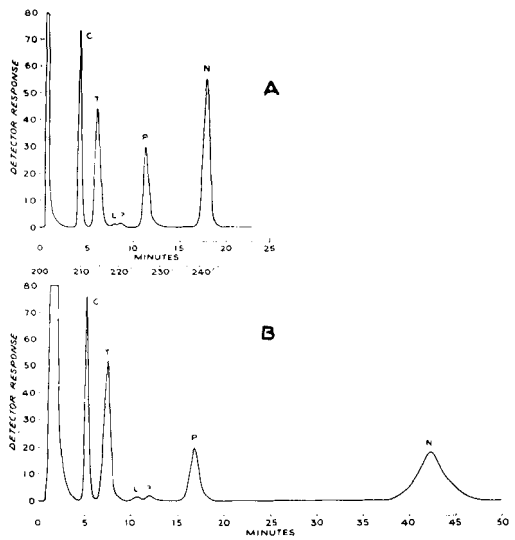


Fig. 5.—Gas chromatograms of the non-phenolic alkaloids of opium on silicone rubber SE-30, 1.15%. A, temperature programing; B, isothermal at 213°. C, codeine; T, thebaine; L, laudanosine; P, papaverine; N, noscapine. Column, 8 feet long and 3 mm. I.D.

operating conditions give two or even three peaks each. One of these appeared to represent the dehydrated alkaloid because the retention value of one of the peaks from atropine was identical with that given by apoatropine prepared according to Hesse (9). The degree of dehydration was found to be associated with the amount of glass wool placed on top of the column packing, and with the temperature of the flash heater. When a fairly large amount of glass wool was used, the degree of decomposition decreased as the flash heater temperature was reduced, but could never be entirely eliminated. When the amount of glass wool was reduced to a very small amount or removed completely, no decomposition took place even at flash heater temperatures of 350°. Similar dehydration of certain steroids has been described as a function of the flash heater temperature by Horning, *et al.* (10).

Hydrolysis and transesterification reactions were also sometimes noticed. Diacetylmorphine was eluted as a sharp peak when chromatographed alone. In mixtures with codeine, morphine or other alcoholic or phenolic substances, reactions taking place in the flash heater gave rise to several new esters not present in the original solution. Both 3, O-monoacetylmorphine (11) and 6, O-monoacetylmorphine[1] gave single peak chromatograms in the absence of glass wool. An apparent catalytic effect of glass wool resulted in peaks corresponding to morphine, monoacetyl- and diacetylmorphine.

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[1] We are indebted to Dr. E. L. Way for supplying us with a sample of this material.

Antiradiation Compounds III

N-2-Mercaptoethylpiperazines

By WILLIAM O. FOYE and DOUGLAS H. KAY

For the purpose of obtaining 2-mercaptoethylamine derivatives having an additional basic function located two carbons distant from the nitrogen, several N-2-mercaptoethylpiperazines have been synthesized. Procedures were employed that involved conversion of the 2-hydroxyethylpiperazines to the corresponding bromo compounds with subsequent thiolation, as well as those employing ethylene sulfide. Both 1-(2′-mercaptoethyl)piperazine and 1,4-bis(2′-mercaptoethyl)piperazine were found to give some protection to mice exposed to 575 roentgens of X-irradiation.

MERCAPTANS have been recognized as effective protective agents against the lethal effects of ionizing radiation, but all of the significantly successful compounds of this variety have so far had a basic function located two or three carbons distant from the sulfhydryl group. The most outstanding examples of the mercaptoamines are 2-mercaptoethylamine (1) and 2-mercaptoethylguanidine (2). Some question remains regarding the basicity of 2-acylthioethylamines, however, which were found to have appreciable antiradiation effects in mice (3), since the acyl groups shift to the nitrogen in neutral or alkaline media (3, 4). Otherwise, a fairly strongly basic group has been required in mercaptans for antiradiation properties, so it appeared desirable to prepare mercaptoamines having an additional basic group located two carbons from the parent amine.

Received March 27, 1962, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.
Accepted for publication May 31, 1962.
This project was carried out under a contract (DA-49-193-MD-2029) with the Office of the Surgeon General, U. S. Army Medical Research and Development Command.
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Previous experience with a series of dithiocarbamates having additional basic groups showed the two carbon distance to be of importance (5). 2-Diethylaminoethyldithiocarbamic acid had radiation-protective ability, while 3-dimethylaminopropyldithiocarbamic acid had none.

For the purpose of providing an additional basic function to the mercaptoamine type of molecule, N-2-mercaptoethylpiperazines have been prepared. In this connection, however, it should be mentioned that N,N-diethylaminoethyl mercaptan is less effective as a radiation-protective agent than 2-mercaptoethylamine (2, 6).

DISCUSSION

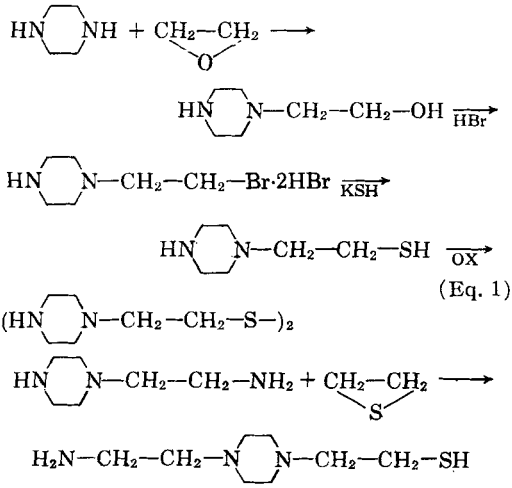
1-(2′-Mercaptoethyl)piperazine was prepared in a conventional manner from the corresponding alcohol. This compound was obtained from the reaction of anhydrous piperazine and ethylene oxide according to McElvain and Bannister (7). This procedure also gave small quantities of the 1,4-bis-alcohol. The latter compound was prepared in quantity, however, by the procedure of Pollard, *et al.* (8). The 1-(2′-hydroxyethyl)piperazine obtained was converted to the corresponding bromo compound by means of 48% hydrobromic acid, a much longer period of refluxing being required than that recommended by McElvain (7), however. Treatment of the 1-(2′-bromoethyl)piperazine dihydrobromide under pressure with a solution of potassium hydroxide saturated with hydrogen sulfide readily yielded the desired mercaptan. This compound was purified under nitrogen and isolated as the dihydrobromide. This reaction sequence is shown in Eq. 1.

The 1,4-bis(2′-hydroxyethyl)piperazine was converted to the bis-mercaptan by the same reaction scheme. The corresponding dibromo compound had been previously reported by McElvain (7), but he listed a bromide analysis that varied from theoretical by several per cent and a melting range of 240–360°. Our product was found analytically acceptable after extraction with hot alcohol. The dibromo compound was converted to the desired bis-mercaptan by treatment under pressure with potassium hydroxide saturated with hydrogen sulfide. It was again necessary to convert the product to the dihydrobromide before isolation was successful.[1]

Ethylene sulfide was employed in an attempt to prepare mercaptoethylpiperazines more conveniently and in better yield. Reaction with anhydrous piperazine in 95% ethanol, however, led only to ethylene sulfide polymer. Reaction in chloroform gave an impure oil that reacted with carbon disulfide to give a solid melting at 55–60°. A satisfactory carbon-hydrogen analysis for the suspected 1-(2′-mercaptoethyl)piperazine-4-carbodithioic acid was not obtained.

Treatment of 1-ethylpiperazine with ethylene sulfide yielded a characterizable product, however. Analysis indicated that the monomercaptoethyl derivative was formed. A lower melting product, from which practically identical analytical values were obtained, was also isolated; this was assumed to be the corresponding disulfide. The low yields obtained may be explained by formation of ethylene sulfide polymer as generally found by Reynolds, *et al.* (10), from reactions with ethylene sulfide.

Reaction of 1-(2′-aminoethyl)piperazine with ethylene sulfide in chloroform also produced a mercaptoethyl derivative for which the analytical results were satisfactory. Infrared absorption showed the presence of a primary amine at 1620 cm.$^{-1}$ so the mercaptoethylation apparently took place on the piperazine nitrogen to give 1-(2′-aminoethyl) - 4 - (2′ - mercaptoethyl)piperazine (see Eq. 2). This was isolated as the trihydrochloride.



(Eq. 1)

(Eq. 2)

Some question remained regarding the structure of the 1-(2′-mercaptoethyl)piperazine. Since this reduced iodine rather slowly, it was suspected that the compound might possibly be the disulfide. However, oxidation of the dihydrochloride of this compound with hydrogen peroxide gave a product of different melting point and with approximately the same analytical values. The latter compound did not reduce iodine and accordingly should be the disulfide.

Biological Activities.—Tests for the ability of three of these compounds to protect mice against X-irradiation have been carried out at the Walter Reed Army Institute of Research under the direction of Dr. D. P. Jacobus. 1,4-Bis(2′-mercaptoethyl) piperazine afforded significant protection to mice when administered 30 minutes prior to exposure to 575 Roentgens. An increase of 65% in 30-day survival over that of the control animals was observed. 1-(2′-Mercaptoethyl)piperazine, however, provided only a 30% increase in 30-day survival. This much lower protective ability may be related to the relative slowness of the compound to reduce iodine and undergo mixed disulfide formation with glutathione (11) in accordance with the mixed disulfide hypothesis of radioprotective action (12). 1 - (2′ - Aminoethyl) - 4 - (2′ - mercaptoethyl)-piperazine, however, provided no protection at the same drug level. None of these compounds provided any protection for mice given a dosage of 800 *r*. A summary of the testing data is given in Table I.

[1] The free base, 1,4-bis-(2′-mercaptoethyl)piperazine, was recently reported by Reynolds, *et al.* (9), from the reaction between piperazine and ethyl 2-mercaptoethylcarbonate.

TABLE I.—ANTIRADIATION PROPERTIES OF N-(2-MERCAPTOETHYL)PIPERAZINES

Compound[a]	Drug Level, mg./Kg.	Adm. Time	Dose, r	30-Day Survival, % Treated	30-Day Survival, % Controls
1-(2′-Mercaptoethyl)piperazine	50	15 min. prior	575	60	30
1,4-Bis-(2′-mercaptoethyl)piperazine	60	30 min. prior	575	95	30

[a] Aqueous solutions of the dihydrobromides were brought to a pH of 7.2 before injection in mice.

EXPERIMENTAL

1-(2′-Hydroxyethyl)piperazine.—Using the procedure of McElvain and Bannister (7), we obtained a 52% yield of product, b.p. 110°(2 mm.). An 8% yield of 1,4-bis(2′-hydroxyethyl)piperazine was also recovered; m.p. 135–136°, which agrees with the reported value (7).

1,4-Bis-(2′-hydroxyethyl)piperazine.—The procedure of Pollard, *et al.* (8), was used, giving an 89% yield of product which melted at 135–136°.

1-(2′-Bromoethyl)piperazine Dihydrobromide.—Twenty grams (0.154 mole) of 1-(2′-hydroxyethyl)-piperazine was cooled in an ice bath and 75 ml. of 48% hydrobromic acid was added slowly, with shaking. The resulting solution was distilled slowly for 30 minutes and the reaction mixture was then refluxed for 8 days.

After a 24-hour reflux period, the presence of 1-(2′-hydroxyethyl)piperazine dihydrobromide was detected by removing a portion of the solution and rubbing it in acetone. A white crystalline solid was obtained which melted at 208–210°. A Volhard bromide determination gave the following result.

Anal.—Calcd. for $C_6H_{16}Br_2N_2O$: Br, 54.79. Found: Br, 54.36.

After 8 days of refluxing, the reaction mixture was evaporated to dryness *in vacuo*. The residue was triturated in acetone to yield approximately 38 Gm. (70%) of product which decomposed at 242–243°, the reported value (13), after recrystallization from 95% ethanol.

1-(2′-Mercaptoethyl)piperazine Dihydrobromide.—A solution containing 2.8 Gm. of 85% potassium hydroxide, 1.5 ml. of distilled water, and 20 ml. of absolute ethanol was saturated with hydrogen sulfide. It was added to 5.0 Gm. (0.014 mole) of 1-(2′-bromoethyl)piperazine dihydrobromide in a 250-ml. pressure bottle which was capped immediately and heated in a water bath for 3 hours with occasional shaking.

After being cooled, the flask contents were evaporated to dryness *in vacuo*. The residue was extracted with 100 ml. of hot absolute ethanol, filtered to remove potassium bromide, and the filtrate was concentrated to about 70 ml. About 35 ml. of ethyl acetate was added to the cooled solution and dry hydrogen bromide was introduced. The solution was stirred and the walls of the flask scratched, and a white, crystalline solid was obtained. After extraction with 95% ethanol, 2.5 Gm. of 1-(2′-hydroxyethyl)piperazine dihydrobromide, m.p. 208–210°, remained. Chilling the ethanolic extract gave 1.3 Gm. (31.6%) of 1-(2′-mercaptoethyl)-piperazine dihydrobromide which decomposed at 220°, with previous softening at 175°.

Anal.—Calcd. for $C_6H_{16}Br_2N_2S$: C, 23.35; H, 5.23. Found: C, 23.50; H, 5.20.

1,4-Bis-(2′-bromoethyl)piperazine Dihydrobromide.—Twenty-five grams (0.144 mole) of 1,4-bis(2′-hydroxyethyl)piperazine was cooled in an ice bath and 100 ml. of 48% hydrobromic acid was added slowly, with shaking. The resulting solution was distilled slowly for 1 hour and was then refluxed for 3 days.

After 24 hours, the reaction mixture was concentrated at 70° (20–30 mm.) and refluxing was resumed. The presence of 1,4-bis(2′-hydroxyethyl)-piperazine dihydrobromide was demonstrated at this time by removing a portion of the solution and rubbing it in acetone. A white, crystalline solid was obtained which melted at 170–185°. A Volhard bromide determination gave the following result.

Anal.—Calcd. for $C_8H_{20}Br_2N_2O_2$: Br, 47.61. Found: Br, 47.20.

After 3 days, the reaction mixture was evaporated *in vacuo* to a syrup. This was triturated in acetone to yield a gummy material; more was obtained by adding ethyl acetate to the acetone extract. The gummy product was extracted with 400 ml. of hot 95% ethanol, and 41 Gm. (62%) of white, crystalline product was obtained which was insoluble in the hot alcohol. The solid decomposed at 230°, which agrees with the reported value (7) for 1,4-bis(2′-bromoethyl)piperazine dihydrobromide.

Anal.—Calcd. for $C_8H_{18}Br_4N_2$: C, 20.81; H, 3.92. Found: C, 21.07; H, 4.05.

1,4-Bis-(2′-mercaptoethyl)piperazine Dihydrobromide.—A solution containing 2.3 Gm. of 85% potassium hydroxide, 1.5 ml. of distilled water, and 15 ml. of absolute ethanol was saturated with hydrogen sulfide. It was added to 4.0 Gm. (0.008 mole) of 1,4-bis-(2′-bromoethyl)piperazine dihydrobromide in a 250-ml. pressure bottle which was capped immediately and heated in a water bath for 3 hours with occasional shaking.

After being cooled, the flask contents were evaporated almost to dryness *in vacuo*. The residue was extracted with 175 ml. of hot absolute ethanol, filtered, and the filtrate was concentrated to about 125 ml. About 30 ml. of ethyl acetate was added and anhydrous hydrogen bromide was introduced into the chilled solution. The solution was chilled and the vessel scratched, and a white, crystalline solid was obtained. The solid was washed with cold ethyl acetate and extracted five times with 200-ml. portions of hot 95% ethanol About 5.0 Gm. (52%) of 1,4-bis-(2′-mercaptoethyl)piperazine dihydrobromide was obtained which was insoluble in the hot alcohol. The compound decomposed at 260°.

Anal.—Calcd. for $C_8H_{20}Br_2N_2S_2$: C, 26.09; H, 5.47. Found: C, 26.24; H, 5.37.

1-Ethyl-4-(2′-mercaptoethyl)piperazine Dihydrochloride.—Ten grams (0.087 mole) of 1-ethylpiperazine (14) was cooled in an ice bath and treated with 5.26 Gm. (0.087 mole) of ethylene sulfide (15) slowly, with shaking, during 20 minutes. The ice bath was removed, and the mixture was allowed to stand overnight at room temperature under nitrogen. It was then refluxed gently for 2 hours and again allowed to stand overnight under

nitrogen. The mixture was evaporated at 60° (20–30 mm.) under nitrogen, and a semisolid was obtained after cooling. This was taken up in 30 ml. of absolute ethanol and cooled, and the solution was saturated with anhydrous hydrogen chloride. A white solid precipitated and after extraction with absolute ethanol, 0.5 Gm. remained which melted at 240–250°, with decomposition beginning at about 210°. The compound contained 11.52% sulfur. From the chilled extract was obtained 0.65 Gm. of solid which melted at 205–207°. This contained 11.55% sulfur. Neither product formed an addition compound with carbon disulfide, and both reduced iodine T.S.

Anal.—Calcd. for $C_8H_{20}Cl_2N_2S$: C, 38.86; H, 8.09; S, 12.95. Found: C, 38.57; H, 8.34; S, 11.52.

1-(2′-Aminoethyl)-4-(2′-mercaptoethyl)-piperazine Trihydrochloride.—A solution of 4.6 Gm. (0.036 mole) of 1-(2′-aminoethyl)piperazine in 30 ml. of chloroform was cooled in an ice bath and treated with ethylene sulfide (4.3 Gm., 0.072 mole) slowly, with shaking, during 30 minutes. The mixture was allowed to stand for 3 days at room temperature under nitrogen, and was then distilled under nitrogen at 40° (20–30 mm.). The residual oil was extracted with two 50-ml. portions of ether, and 125 ml. of absolute ethanol was added to the ether-insoluble material. The alcohol solution was chilled and saturated with anhydrous hydrogen chloride; a solid was obtained which was extracted with 100 ml. of hot acetone in two portions and quickly transferred to a desiccator. About 2.5 Gm. (24%) of ivory-colored product was obtained which melted at 165–170°.

Anal.—Calcd. for $C_8H_{22}Cl_3N_3S$: C, 32.15; H, 7.42; S, 10.71. Found: C, 32.52; H, 7.72; S, 10.94.

1-(2′-Mercaptoethyl)piperazine Dihydrochloride.—The same reaction sequence was used as in the preparation of the corresponding dihydrobromide. A very small yield of hygroscopic product was obtained which melted in the range of 145–150°.

Anal.—Calcd. for $C_6H_{16}Cl_2N_2S_2 \cdot H_2O$: C, 30.37; H, 7.65; N, 11.81. Found: C, 30.15; H, 7.51; N, 10.49.

1-Piperazinoethyl Disulfide Tetrahydrochloride.—The previous product, 1.0 Gm., was dissolved in 5 ml. of 50% aqueous ethanol containing 5 ml. of 3% hydrogen peroxide solution. The solution was heated on a water bath for 30 minutes and allowed to stand overnight. The solution was evaporated *in vacuo* to a gum, treated with absolute ethanol, and again concentrated *in vacuo*. This process was repeated. The semicrystalline material was removed from the flask and triturated in absolute ethanol; an extremely hygroscopic product was isolated which did not decolorize iodine T.S. without remaining in solution for some time. The product, 0.7 Gm., melted at 95–100°.

Anal.—Calcd. for $C_{12}H_{30}Cl_4N_4S_2 \cdot 2H_2O$: C, 30.51; H, 7.25; N, 11.86. Found: C, 29.90; H, 7.19; N, 10.20.

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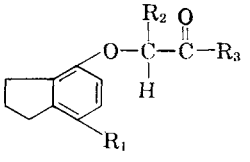
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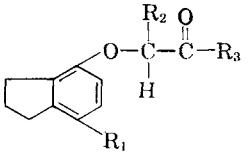
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nitrogen. The mixture was evaporated at 60° (20–30 mm.) under nitrogen, and a semisolid was obtained after cooling. This was taken up in 30 ml. of absolute ethanol and cooled, and the solution was saturated with anhydrous hydrogen chloride. A white solid precipitated and after extraction with absolute ethanol, 0.5 Gm. remained which melted at 240–250°, with decomposition beginning at about 210°. The compound contained 11.52% sulfur. From the chilled extract was obtained 0.65 Gm. of solid which melted at 205–207°. This contained 11.55% sulfur. Neither product formed an addition compound with carbon disulfide, and both reduced iodine T.S.

Anal.—Calcd. for $C_8H_{20}Cl_2N_2S$: C, 38.86; H, 8.09; S, 12.95. Found: C, 38.57; H, 8.34; S, 11.52.

1-(2′-Aminoethyl)-4-(2′-mercaptoethyl)-piperazine Trihydrochloride.—A solution of 4.6 Gm. (0.036 mole) of 1-(2′-aminoethyl)piperazine in 30 ml. of chloroform was cooled in an ice bath and treated with ethylene sulfide (4.3 Gm., 0.072 mole) slowly, with shaking, during 30 minutes. The mixture was allowed to stand for 3 days at room temperature under nitrogen, and was then distilled under nitrogen at 40° (20–30 mm.). The residual oil was extracted with two 50-ml. portions of ether, and 125 ml. of absolute ethanol was added to the ether-insoluble material. The alcohol solution was chilled and saturated with anhydrous hydrogen chloride; a solid was obtained which was extracted with 100 ml. of hot acetone in two portions and quickly transferred to a desiccator. About 2.5 Gm. (24%) of ivory-colored product was obtained which melted at 165–170°.

Anal.—Calcd. for $C_8H_{22}Cl_3N_3S$: C, 32.15; H, 7.42; S, 10.71. Found: C, 32.52; H, 7.72; S, 10.94.

1-(2′-Mercaptoethyl)piperazine Dihydrochloride.—The same reaction sequence was used as in the preparation of the corresponding dihydrobromide. A very small yield of hygroscopic product was obtained which melted in the range of 145–150°.

Anal.—Calcd. for $C_6H_{16}Cl_2N_2S_2 \cdot H_2O$: C, 30.37; H, 7.65; N, 11.81. Found: C, 30.15; H, 7.51; N, 10.49.

1-Piperazinoethyl Disulfide Tetrahydrochloride.—The previous product, 1.0 Gm., was dissolved in 5 ml. of 50% aqueous ethanol containing 5 ml. of 3% hydrogen peroxide solution. The solution was heated on a water bath for 30 minutes and allowed to stand overnight. The solution was evaporated *in vacuo* to a gum, treated with absolute ethanol, and again concentrated *in vacuo*. This process was repeated. The semicrystalline material was removed from the flask and triturated in absolute ethanol; an extremely hygroscopic product was isolated which did not decolorize iodine T.S. without remaining in solution for some time. The product, 0.7 Gm., melted at 95–100°.

Anal.—Calcd. for $C_{12}H_{30}Cl_4N_4S_2 \cdot 2H_2O$: C, 30.51; H, 7.25; N, 11.86. Found: C, 29.90; H, 7.19; N, 10.20.

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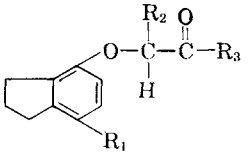
ERRATUM

In the paper titled "The Pharmacologic Effects of a New Analgesic α-4-dimethylamino-1,2-diphenyl-3-methyl-4-propionyloxybutane" (1), the analgesic should have been identified as α-4-dimethylamino-1,2-diphenyl-3-methyl-2-propionyloxybutane.

(1) Robbins, E. B., THIS JOURNAL, **44**, 497(1955).

ERRATUM

In the paper titled "Indanols V. Indanoxyacetic Acid Derivatives" (1), Table I should have included the structure heading



between compounds 41 and 42 to indicate that the second part of the Table referred to 7-substituted-4-indanols.

(1) Shapiro, S. L., Bazga, T., and Freedman, L., THIS JOURNAL, **51**, 582(1962).

Soluble Steroids I

Sugar Derivatives

By WINTHROP E. LANGE and MERLE E. AMUNDSON†

Glucamine, glucosamine, and N-methylglucamine derivatives of cholesterol, testosterone, 9-α-fluorohydrocortisone, triamcinolone acetonide, and hexestrol have been synthesized in an attempt to provide compounds with increased aqueous solubility. The acylable hydroxy group of the steroid was converted to the chloroformate and then condensed with the appropriate amino sugar. In general, the derivatives which have been prepared possess 8 to 13 times the aqueous solubility of the parent compounds. The N-methylglucamine and glucosamine derivatives of triamcinolone acetonide, when applied topically, failed to show appreciable pharmacological activity.

THERE HAVE been two general methods for the preparation of water-soluble steroidal compounds. One method is by means of physical factors, that is, solubilizing agents which have been used to increase the solubility of the steroid. The second method for the preparation of water-soluble steroidal compounds has been by modification of structure. Of the many modifications that have been reported, the preparation of poly-hydroxy derivatives have been mentioned in only a few cases. Water-soluble compounds, useful as sedatives showing no hormonal activity, have been prepared by the formation of glycosides of 21-hydroxysteroids and of hemiglutarates and hemiphthalates (1, 2). A patent granted to the Upjohn Co. (3) describes physiologically active water-soluble glycoside derivatives of corticoid hormones. One of the compounds prepared was the N-methylglucamine salt of hydrocortisone-21-hemi-β-methylglutarate.

Nakagawa and Mori (4) prepared methoxy poly-(oxyethylene)-propionic acid and obtained water-soluble esters with various steroids.

Plungian (5) reported the preparation of a stable, nontoxic complex of rutin and N-methylglucamine (3:1), which retained the therapeutic properties of rutin. This complex was soluble in water to the extent of 20% and had a pH suitable for oral or parenteral preparations.

These various findings suggested then that a sugar moiety attached to a steroid nucleus might give compounds with increased water solubility. The poly-hydroxy chain would be so attached to the steroid nucleus that it would be readily hydrolyzed in the body, a condition which would allow the free steroid to exert its full pharmacological effect. It is also possible that such derivatives should have some effect upon the absorption and distribution of the drug in the body. Thus, the present investigation is intended to make available for testing various steroids containing sugar moieties, such as glucamine, N-methylglucamine, and glucosamine.

DISCUSSION

Several methods have been reported for the preparation of amine derivatives of steroids. Pierce and co-workers (6) prepared a series of steroidal amines by direct replacement of halogen or tosylate groups with an amine group.

Heyl and Herr (7) prepared a series of C_3-(N-pyrrolidyl)-enamines (α,β-unsaturated amines) from steroidal C_3-ketones by azeotropic distillation with pyrrolidine. The enamines were readily hydrolyzable to regenerate the carbonyl function. Using this method, a model synthesis for application to steroids was attempted with cyclohexanone. Cyclohexanone was refluxed with N-methylglucamine in benzene with constant water separation. The compound prepared, N-methyl-N-(1-cyclohexenyl)-glucamine, was completely water soluble. However, when Δ^4-cholestenone-3 was chosen as a representative steroid, the enamine was not obtained. Several modifications were also attempted without success. The reactants were refluxed in solvents other than benzene, dehydrating agents were added, the steroid was changed to testosterone, and the sugar was changed to 2,3,4,5,6-pentaacetyl-N-methyl glucamine. The application of the Leukart reductive amination process (8) to Δ^4-cholestenone-3 and the direct reaction of cholesteryl chloride with a sugar amine also were unsuccessful.

The report of Verdino and Schadendorff (9) that amines could be condensed with cholesterol chloroformate to give carbamates, and the finding of Miescher and Scholz (10) that carbonates of 17-estradiol had increased physiological action, suggested a second method for the attachment of amino sugars to steroids.

Cholesterol was converted to 5-cholesten-3β-yl-chloroformate by the method of Wieland, Honold

Received March 27, 1962, from the Samuel M. Best Research Laboratories of the Massachusetts College of Pharmacy, Boston.

Accepted for publication May 17, 1962.

Abstracted in part from the dissertion presented by Merle E. Amundson to the Graduate Council, the Massachusetts College of Pharmacy, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The authors are deeply indebted to Dr. L. V. Blubaugh for his generous donation of several of the steroids and arranging for the pharmacological screening of the sugar derivatives.

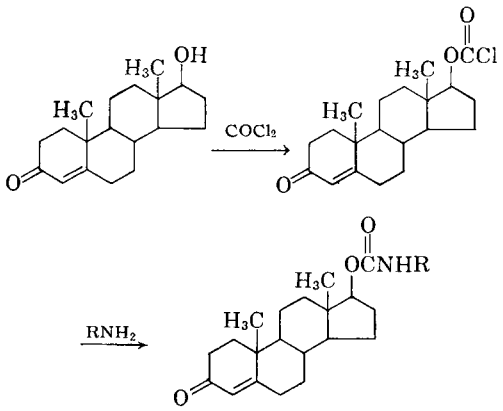
Presented to the Scientific Section, A.PH.A. Las Vegas meeting, March 1962.

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and Pascual-Vila (11) in quantitative yields. The chloroformate was then refluxed with N-methylglucamine in a dioxane-water mixture. The reaction was completed in 1 hour and the product, 3β-(5-cholesten-N-methyl-N-1-(1-desoxyglucosyl) carbamate was isolated. Carbon-hydrogen analysis on recrystallized samples of this compound repeatedly resulted in low results, and it was only after the compound had been dried 2 hours at 105–110° that a satisfactory analysis was obtained. A water analysis showed the presence of 1 mole of water per mole of compound in the sample dried at 56° *in vacuo*.

5-Cholesten-3β-yl-chloroformate was also coupled with glucosamine, but only gave a gummy product with glucamine.

The product obtained after the treatment of 9α-fluorohydrocortisone with phosgene contained only one chloroformyl group. Thus, it was desirable to determine the position of condensation since hydroxyl groups are present at positions 11, 17, and 21. Steric considerations make it practically impossible for reaction to occur with the 11β-hydroxyl group. The two strong methyl:hydroxyl repulsions and one hydroxyl:hydrogen interaction place the molecule under considerable strain. Since these factors would not be relieved by chloroformyl formation, the 11β-hydroxyl group is practically unacylable. In addition, an equatorial hydroxyl group is acylated more readily than an axial group at the same position and, in this case, the hydroxyl groups at the 11 and 17 positions are axial. Final evidence that the 21 position is involved was obtained when phosgene was introduced into a tetrahydrofuran solution of 9α-fluorohydrocortisone-21-acetate and no chloroformate was obtained. Only the parent substance, the acetate, was isolated.



R = Sugar Moiety

Triamcinolone acetonide also gave only a monochloroformate. However, with this compound as well as with the 9α-fluorohydrocortisone all three sugar derivatives were obtained. The general reaction for the preparation of the sugar derivatives is summarized by the following equation and the analytical data are given in Table I.

An attempt was also made to prepare the sugar derivatives of diethylstilbestrol as it is a representative of that class of steroids and steroid-like materials which contain a phenolic hydroxyl group. The method of Crosby and Niemann (12) for the preparation of chloroformates of phenol with diethylstilbestrol gave, on one attempt, a product with the properties of a chloroformate. However, a carbon-hydrogen analysis did not agree with the calculated values for the chloroformate. Successive trials, and even modifications in the procedure as suggested by Dirschel (13) were of no avail in the preparation of the chloroformate of diethylstilbestrol.

When hexestrol, however was dissolved in aqueous sodium hydroxide and a cooled solution of phosgene in benzene was added, a good yield of 3,4-bis-(*p*-chloroformylphenyl)-*n*-hexane was obtained. Sugar derivatives of hexestrol chloroformate were then prepared in the usual manner. The hydroscopic nature of the chloroformate and sugar derivatives of hexestrol presented difficulty in obtaining analytically pure samples.

Absorption Data.—The ultraviolet spectra of the

TABLE I.—CHLOROFORMATE AND SUGAR DERIVATIVES OF SELECTED STEROIDS

Compound	M.p., °C.[a]	Yield, %	Formula	Analyses, %[b] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
Cholesterol-NMG[c]	131–133	90	$C_{35}H_{61}NO_7$	69.15	68.38	10.12	9.79
			$C_{35}H_{61}NO_7 \cdot H_2O$	67.16	66.98	10.15	10.14
Cholesterol-GLS[c]	155–158	63	$C_{34}H_{57}NO_7$	69.00	68.70	9.71	9.53
			$C_{34}H_{57}NO_7 \cdot H_2O$	67.01	67.07	9.75	9.66
Testosterone-CF[c]	110–111	95	$C_{20}H_{27}ClO_3$	68.46	68.72	7.75	7.76
Testosterone-NMG	183–185	95	$C_{27}H_{43}NO_8$	63.63	63.27	8.50	8.68
Testosterone-GLS	185–190[d]	89	$C_{26}H_{38}NO_8$	63.24	63.09	7.75	8.18
			$C_{26}H_{38}NO_8 \cdot H_2O$	61.04	61.98	7.88	8.14
9α-Fluorohydrocortisone-CF	159–162	95	$C_{22}H_{28}ClFO_6$	59.66	59.66	6.37	6.96
9α-Fluorohydrocortisone-NMG[e]	120[f]	52	$C_{29}H_{44}FNO_{11} \cdot H_2O$	56.21	55.71	7.48	7.45
9α-Fluorohydrocortisone-GLS[e]	176–178	68	$C_{28}H_{40}FNO_{11} \cdot H_2O$	55.73	55.35	7.01	6.62
9α-Fluorohydrocortisone-GLC[c,e]	105–110	54	$C_{28}H_{42}FNO_{11} \cdot H_2O$	55.53	55.93	7.32	6.97
Triamcinolone acetonide-CF	165–167	90	$C_{25}H_{30}ClFO_7$	60.44	60.17	6.08	5.73
Triamcinolone acetonide-NMG[e]	152[g]	38	$C_{32}H_{46}FNO_{12} \cdot H_2O$	57.05	57.22	7.18	6.34
Triamcinolone acetonide-GLS[e]	250–255[d]	98	$C_{31}H_{42}FNO_{12} \cdot H_2O$	56.61	56.59	6.74	6.74
Triamcinolone acetonide-GLC[e]	150[d]	83	$C_{31}H_{44}FNO_{12} \cdot H_2O$	56.44	55.58	7.03	6.94
Hexestrol-CF	115–117	66	$C_{20}H_{20}Cl_2O_4$	60.77	62.81	5.10	5.40
Hexestrol-NMG	80–82	28	$C_{34}H_{52}N_2O_{14}$	57.29	59.55	7.35	7.11
Hexestrol-GLS	188–190	15	$C_{32}H_{44}N_2O_{14}$	56.46	56.21	6.52	6.65

[a] Melting points are not corrected. [b] Analyses by Weiler and Strauss, Oxford, England, and by Carol K. Fitz, Needham Heights, Mass. [c] NMG is N-methylglucamine, GLS is glucosamine, GLC is glucamine, and CF is chloroformate. [d] With decomposition. [e] These compounds possess a molecule of water which is not driven off at 105–110°. [f] Softened only. [g] With previous softening.

sugar derivatives which have been described parallel those of the parent compounds, and no new maxima are exhibited.

Several significant changes appear, however, in the infrared spectra of the derivatives in the range from 2.5 to 6.5 μ. The 2.8 or 2.9 band, which is attributed to the hydroxyl group, is not found in the spectrum of the chloroformates but is present in the sugar derivatives of cholesterol, testosterone, and hexestrol.

The carbonyl band of the chloroformyl radical exhibits a maximum between 5.60 and 5.62 μ for all of the steroids. The carbonyl band for the substituted carbamates exhibits a maximum between 5.84 and 5.90 μ. The glucosamine and glucamine derivatives also show a new band between 6.44 and 6.58 μ which is attributed to the amide II band of secondary amides or secondary carbamates. Thus, this band furnishes evidence that the sugar derivatives are carbamates and not carbonates. The characteristic maxima of all compounds are found in Table II.

Although a pure chloroformate of diethylstilbestrol was not obtained, spectral evidence was obtained for the formation of such a compound.

Solubility Studies.—Since the primary objective of this project was an attempt to prepare a series of steroidal derivatives which would have an increased solubility in water over the parent steroids, a solubility study was undertaken. Approximately 25 mg. of product was accurately weighed and placed in a 50-ml. Erlenmeyer flask which contained exactly 25 ml. of distilled water. The flask was stoppered and placed on a mechanical shaker for 24 hours at 25°. At the end of this time the contents of the flask were filtered by means of a tared sintered-glass funnel. The insoluble material which collected on the funnel was dried to constant weight in a desiccator over anhydrous calcium chloride. The funnel and the contents were then accurately weighed and the amount of product which dissolved was determined by difference. The results of this study are summarized in Table III.

It will be noted that the increase in solubility of the various sugar derivatives of the steroids follows a pattern when steroids substituted at the same position are compared. However, when a 3-hydroxy steroid is compared with a 21-hydroxy steroid, the solubilizing effect of a particular sugar moiety does not follow a pattern as would be expected. No explanation for these results is available at this time.

Preliminary Hydrolysis Study.—It was desirable to obtain information regarding the rate of hydrolysis of the sugar derivatives in order to predict their stability characteristics.

Using the N-methylglucamine derivative of cholesterol it was noted that no measureable hydrolysis occurred after 7 hours in either U.S.P. XVI simulated gastric or intestinal fluid at 37°. However, after 4 hours in solutions of 1 *N* sodium hydroxide and 1 *N* hydrochloric acid, 13% of the sugar derivative in the alkaline solution had hydrolyzed, while no hydrolysis was noted in the acid solution.

Hydrolysis was detected in the aqueous solutions by extracting them with ether and isolating the cholesterol which was released.

Preliminary Pharmacological Results.—At the present time, two of the compounds (N-methylglucamine and glucosamine derivatives of triamcinolone) have been tested at the Squibb Institute for Medical Research. The steroids were assayed in adrenalectomized male rats. In liver glycogen studies the N-methylglucamine derivative was approximately 10 times as active as cortisone acetate, while the glucosamine derivative was only 2.5 times as active. In antigranuloma (pellet) and thymolytic tests, the sugar derivatives were about 0.1 as active as the parent compound. It appears from these tests that the sugar moiety stabilizes the carbamate linkage to hydrolysis with the resultant loss in activity.

EXPERIMENTAL

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

***N*-Methyl-*N*-1-(1-cyclohexenyl)glucamine.**—Cyclohexanone (National Aniline), 10.0 Gm. (0.1 mole), was dissolved in 50 ml. of dried benzene. N-Methylglucamine (Aldrich Chemical Co.), 19.52 Gm. (0.1 mole), was added to the benzene solution and the resulting mixture was refluxed for 5 hours with constant water separation by means of a Bidwell-Sterling moisture trap. The insoluble material was then filtered, dried, and recrystallized from a benzene-methanol mixture. A quantitative yield of white needles which melted at 93–95° was obtained.

Anal.—Calcd. for $C_{13}H_{25}NO_5$: C, 56.7; H, 9.1. Found: C, 56.4; H, 7.9.

2,3,4,5,6-Pentaacetyl-*N*-methylglucamine Hydrochloride.—N-Methylglucamine hydrochloride, 15 Gm. (0.064 mole), was placed in 75 ml. of acetic anhydride, and five drops of concentrated sulfuric acid was slowly added to the mixture. The mixture was heated 2 hours on a steam bath, cooled, and refrigerated overnight. The product was precipitated from solution by the addition of several volumes of ether and was collected on a filter. The white amorphous product was washed several times with ether and stored under anhydrous conditions in a refrigerator. The product weighed 22.8 Gm. (80%) and melted at 90–92° with previous softening.

Anal.—Calcd. for $C_{17}H_{27}NO_{10}\cdot HCl$: C, 46.20; H, 6.39. Found: C, 45.29; H, 6.17.

5-Cholesten-3β-yl-chloroformate.—This compound was prepared according to the method of Wieland, Honold, and Pascual-Vila (11). A yield of 79% was obtained which melted at 121–122°. This melting point agrees with the reported value.

3β-(5-Cholestenyl)-N-methyl-N-1-(1-desoxyglucosyl)carbamate.—To 9 Gm. (0.02 mole) of 5-cholesten-3β-yl-chloroformate dissolved in 100 ml. of dioxane was added 8.0 Gm. (0.04 mole) of N-methylglucamine and sufficient water to obtain a clear solution. The mixture was refluxed for 1 hour and was then cooled and refrigerated. The product was collected on a filter and recrystallized from ethanol. A yield of 11.0 Gm. of white needles was obtained which melted at 131–133°.

Anal.—Calcd. for $C_{35}H_{61}NO_7\cdot H_2O$: H_2O, 2.88. Found: H_2O, 2.70.

3β-(5-Cholestenyl)-N-2-(2-desoxyglucosyl)-carbamate.—5-Cholesten-3β-yl-chloroformate, 4.5 Gm. (0.01 mole), was dissolved in 60 ml. of dioxane with slight warming. D(+) Glucosamine hydro-

TABLE II.—INFRARED ABSORPTION CHARACTERISTICS

Compound	Absorption Maxima, μ[a]						
Cholesterol	2.80	...	...	...	...	...	...
Cholesterol-CF[b]	...	...	5.62	...	...	...	...
Cholesterol-NMG[b]	2.86	2.96	...	5.93	...	...	...
Cholesterol-GLS[b]	2.92	...	...	5.90	...	...	6.50
Testosterone	2.90	...	...	5.95	...	...	...
Testosterone-CF	...	...	5.60	5.95	...	...	...
Testosterone-NMG	3.00	...	5.90	5.95	...	...	...
Testosterone-GLS	2.85	...	...	5.95	...	...	6.58
9α-Fluorohydrocortisone	2.70	2.90	...	5.80	6.01	6.15	...
9α-Fluorohydrocortisone-CF	2.75	2.85	5.60	5.80	6.03	6.18	...
9α-Fluorohydrocortisone-NMG	...	2.90	5.80	5.90	6.02	...	...
9α-Fluorohydrocortisone-GLS	...	2.90	5.80	...	6.08	...	6.50
9α-Fluorohydrocortisone-GLC[b]	...	2.88	5.80	5.98	...	...	6.50
Triamcinolone acetonide	2.90	...	...	5.86	6.02	6.19	...
Triamcinolone acetonide-CF	2.82	...	5.62	5.75	6.00	6.16	...
Triamcinolone acetonide-NMG	2.92	...	...	5.84	6.02	6.16	...
Triamcinolone acetonide-GLS	2.90	...	...	5.80	6.02	6.17	6.52
Triamcinolone acetonide-GLC	2.82	...	5.72	5.92	6.08	6.16	6.45
Diethylstilbestrol	2.85	...	...	...	...	6.23	...
Diethylstilbestrol-CF	...	...	5.58	...	...	6.23	...
Hexestrol	2.90	...	...	...	6.22	6.30	...
Hexestrol-CF	...	...	5.60	...	...	6.30	...
Hexestrol-NMG	2.88	...	...	5.93	6.23	6.30	...
Hexestrol-GLS	2.93	...	...	5.88	...	...	6.44

[a] All determinations were made on a Perkin-Elmer model 137 Infracord spectrophotometer. Samples were mounted as Nujol mulls. [b] CF is chloroformate, NMG is N-methylglucamine, GLS is glucosamine, and GLC is glucamine.

chloride (Nutritional Biochemicals Corp.), 4.31 Gm. (0.02 mole), was placed in 20 ml. of distilled water and 0.80 Gm. (0.02 mole) of sodium hydroxide was added. The neutralized aqueous solution of the sugar was then added to the dioxane solution, and the mixture was heated for 10 minutes on a hot plate. The mixture was then shaken for 1 hour and the precipitate which formed was collected on a filter, washed with acetone, and dried *in vacuo* at 56°. A yield of 3.75 Gm. was obtained, which melted at 155–158°.

Anal.—Calcd. for $C_{34}H_{57}NO_7 \cdot H_2O$: H_2O, 2.95. Found: H_2O, 3.26.

4-Androsten-3-one-17β-yl-chloroformate.—To a solution of 5.0 Gm. (0.0175 mole) of testosterone (Matheson, Coleman, and Bell) dissolved in 100 ml. of benzene was added an excess of phosgene. The mixture was allowed to stand 1 hour, after which the solution was aerated for 2 hours. The benzene was evaporated *in vacuo* and the residue recrystallized from Skelly B. A yield of 5.8 Gm. of yellow needles, which melted at 110–111°, was obtained. The melting point has previously been reported as 139–140° (14).

Anal.—Calcd. for $C_{20}H_{27}ClO_3$: C, 68.46; H, 7.75. Found: C, 68.72; H, 7.76.

17β-(4-Androsten-3-one)-N-methyl-N-1-(1-desoxyglucosyl)carbamates.—In a manner similar to that described for the preparation of the N-methylglucamine derivative of cholesterol, using an acetone-water mixture as the solvent and a 3-hr. reflux time, there was obtained a white product. The product melted at 183–185° after recrystallization from aqueous methanol.

17β-(4-Androsten-3-one)-N-2-(2-desoxyglucosyl)carbamate.—This was prepared by the procedure given for the condensation of glucosamine with cholesterol using an acetone-water solvent

TABLE III.—SOLUBILITY DATA

Compound	Solubility,[a] mg./100 ml.	Solubility Ratio[b]
Cholesterol	5.2	...
Cholesterol-NMG[c]	11.2	2.2
Cholesterol-GLS[c]	56.4	10.8
Testosterone	4.8	...
Testosterone-NMG	4.4	0.9
Testosterone-GLS	65.6	13.7
9α-Fluorohydrocortisone	32.4	...
9α-Fluorohydrocortisone-NMG	394.0	12.3
9α-Fluorohydrocortisone-GLS	36.0	1.1
9α-Fluorohydrocortisone-GLC[c]	269.6	8.3
Triamcinolone acetonide	26.4	...
Triamcinolone acetonide-NMG	319.6	12.1
Triamcinolone acetonide-GLS	37.6	1.4
Triamcinolone acetonide-GLC	354.0	13.4
Hexestrol	Insoluble[d]	...
Hexestrol-NMG	Soluble	++
Hexestrol-GLS	Slightly soluble	+

[a] The solubilities of the compounds in water at 25° were determined by a gravimetric procedure following agitation for 24 hours. [b] Solubility of the derivative/solubility of the parent steroid. [c] NMG is N-methylglucamine, GLS is glucosamine, and GLC is glucamine. [d] Exact solubilities were not determined for hexestrol and its sugar derivatives due to the limited amount of material which was obtained in synthesis.

system. The product melted at 185–190°, with decomposition.

9α - Fluoro - 11β,17α - dihydroxy - 4 - pregnen-3,20 - dione - 21 - chloroformate.—The 9α - fluorohydrocortisone (E. R. Squibb and Co.) was treated with phosgene in tetrahydrofuran, as previously described, to give a waxy, light brown compound which melted at 159–162°.

21 - (9α - Fluoro - 11β,17α - dihydroxy - 4-pregnen - 3,20 - dione) - N - methyl - N - 1 - (1-desoxyglucosyl)carbamate.—This was prepared by the procedure given previously for N-methylglucamine derivatives. The light brown product softened at 120°.

21 - (9α - Fluoro - 11β,17α - dihydroxy - 4-pregnen - 3,20 - dione) - N - 2 - (2 - desoxyglucosyl)-carbamate.—This was prepared by the procedure given previously for glucosamine derivatives. The product melted at 176–178°.

21 - (9α - Fluoro - 11β,17α - dihydroxy - 4-pregnen - 3,20 - dione) - N - 1 - (1 - desoxyglucosyl)-carbamate.—The glucamine was prepared according to the method of Holly, *et al.* (15). The derivative was prepared by the procedure given previously for N-methylglucamine. The product after recrystallization from aqueous methanol, melted at 128–130°.

9α - Fluoro - 11β - hydroxy - 16α,17α - isopropylidenedioxy - 1,4 - pregnadien - 3,20 - dione - 21-chloroformate.—The triamcinolone acetonide (E. R. Squibb and Co.) was treated with phosgene in dioxane. The yellow waxy product, after recrystallization from aqueous acetone, melted at 165–167°.

21 - (9α - Fluoro - 11β - hydroxy - 16α,17α - isopropylidenedioxy - 1,4 - pregnadien - 3,20 - dione)-N - methyl - N - 1 - (1 - desoxyglucosyl)carbamate.—This was prepared by the procedure given previously for N-methylglucamine derivatives. Purification of the product was accomplished by treating a warm acetone solution of the compound with charcoal. The yellow product, after drying for 2 hours at 110°, melted at 152° with previous softening.

21 - (9α - Fluoro - 11β - hydroxy - 16α,17α - isopropylidenedioxy - 1,4 - pregnadien - 3,20 - dione)-N - 2 - (2 - desoxyglucosyl)carbamate.—This was prepared by the procedure given previously for glucosamine derivatives. Purification was accomplished in the same manner as described above. The product melted at 250–255°, with decomposition.

21 - (9α - Fluoro - 11β - hydroxy - 16α,17α - isopropylidenedioxy - 1,4 - pregnadien - 3,20 - dione)-N - 1 - (1 - desoxyglucosyl)carbamate.—This was prepared according to the procedure given previously for N-methylglucamine derivatives. The product recrystallized from aqueous methanol melted at 150°, with decomposition.

3,4 - Bis - (*p* - chloroformylphenyl) - *n* - hexane.—Hexestrol, 0.54 Gm. (0.002 mole), (Nutritional Biochemicals Corp.) dissolved in 100 ml. of 2.5% aqueous sodium hydroxide was added, dropwise, to a cooled solution of 1.0 Gm. of phosgene in 75 ml. of benzene. The mixture was stirred for 3 hours at room temperature. The benzene layer was separated and was washed with two volumes of 2.5% aqueous sodium hydroxide and one volume of water. The benzene solution was dried and evaporated to give 0.52 Gm. of white crystals which melted at 115–117°.

3,4 - Bis - {*p* - [N - methyl - N - 1 - (1 - desoxyglucosyl)carbamoylphenyl]} - *n* - hexane.—This was prepared by the procedure given previously for N-methylglucamine derivatives. The syrupy residue which was obtained was dried *in vacuo* at 56°. The hygroscopic product melted at 80–82°.

3,4 - Bis - {*p* - [N - 2 - (2 - desoxyglucosyl)-carbamoylphenyl]} - *n* - hexane.—This was prepared by the procedure given previously for glucosamine derivatives. Purification was effected in an acetone solution by treatment with charcoal. The product melted at 188–190°.

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Treatment of Primary and Secondary Infections of the Skin with Cetyltrimethylammonium Bromide

By H. H. RODIN

Cetyltrimethylammonium bromide incorporated in an aromatic mix base is effective in the treatment of primary and secondary pyogenic cutaneous infections. The failure of this preparation to produce allergic reactions suggests a low sensitizing index.

IN THIS investigation cetyltrimethylammonium bromide (CETAB) incorporated in a water soluble base containing an aromatic mix[1] was found to be effective antimicrobial agent in the treatment of primary skin infections. It has also been found to be effective in the elimination of complicating secondary infections of various skin conditions thus permitting proper treatment of the primary condition.

METHODS AND TECHNIQUES

This study was conducted in the out-patient dermatological clinic of a large mid-western county hospital and is based on observations made on 100 patients treated for superficial pyodermic infections of the skin with a cream containing CETAB and an aromatic mix.

In this series there were 21 white male subjects; 38 male colored subjects; 9 white female subjects and 32 colored female subjects. The youngest patient treated was a 2-year old colored female child and the oldest a 79-year old white female patient.

Treatment consisted of having the subjects apply the medication to the involved areas at least twice daily. In the more severe cases it was applied as often as three or four times daily. Although a few cases were treated shortly after the onset of the disease, less than 1 week, the majority of cases were subacute or chronic and had been present for 2 weeks to 6 months or longer. As a result, many of the cases presented disseminated lesions.

There were 75 subjects who presented six different types of primary skin infections and 25 subjects who presented secondary infections complicating 10 different types of common dermatological disorders. The diagnosis, order of frequency, and results are listed in Table I. The period of treatment in the majority of cases was 7 days, although several cases were treated for 2 weeks, and in a few cases, therapy was continued for 1 month. All clinical evaluations were made at the end of 1 week, regardless of the duration of treatment, in order to maintain the evaluation of the therapeutic index as constant as possible.

Cultures were taken from the lesions of 14 patients selected at random by the dry swab technique. These were transported within a short time (1–2 hr.) to the bacteriological laboratory where they were transferred to blood-agar plates and incubated for 48 to 72 hours. The organisms were then identified and reported by the director of the laboratory.

Of 75 patients treated for primary skin infections, 53 (70.6%) obtained satisfactory results (estimated improvement 50% or better), 15 (20%) obtained fair results (estimated improvement 25% to 50%) while 7 failed to obtain any benefit from therapy.

In those patients treated for dermatological conditions complicated by secondary infection 15 patients (60%) obtained satisfactory results while 10 patients obtained only partial benefit from this form of therapy.

Culture.—An analysis of the cultures revealed that hemolytic *Staphylococcus aureus coagulase positive* was obtained as the only infecting microorganism in six cases and that both it and hemolytic *Staphylococcus albus* were present in five cases. The culture in one case of folliculitis of the scalp revealed a neisseria organism along with an unidentified yeast, while in two cases no growth was reported initially. One of these two cases failed to show growth on repeated culture.

Of the six patients from whom only hemolytic *Staphylococcus aureus coagulase positive* was recovered, four obtained good results and two obtained fair results from the treatment with CETAB cream.

In those cases in which both hemolytic *Staphylococcus aureus coagulase positive* and hemolytic *Staphylococcus albus* were recovered, three patients obtained good results, one patient fair results while one patient failed to obtain any benefit from therapy. The one case in which the neisseria organism and yeast were recovered obtained an excellent result from treatment. In the two cases with negative findings, one obtained excellent results from therapy and the other only fair results.

Reactions.—No clinical evidence of allergic or primary irritant reactions were noted in any of the cases treated for primary skin infections or secondary infections complicating pre-existing dermatological conditions where active treatment was indicated. In several cases, patients complained of a slight transient burning sensation when the medication was first applied. This burning sensation usually disappeared in a matter of seconds and in no instance interfered with the continuation of treatment or affected clinical results. These results suggest that CETAB cream when used properly and in indicated conditions[2] has a low sensitizing and irritant index.

Received December 20, 1961, from 822 Oakridge Dr., South Bend 17, Ind.
Accepted for publication March 16, 1962.

[1] Furnished as Bactine cream, Miles Laboratories, Inc., Elkhart, Ind.

[2] Like all antimicrobial agents, CETAB cream will produce irritation when applied to those cases which are complicated by superimposed acute dermatitis venanata, regardless of cause, and when such active treatment is contraindicated.

TABLE I.—RESULTS OF THERAPY

Diagnosis	No. Cases	Excellent	Good	Fair	No Change
Primary Skin Infections					
Pyoderma	25	4	12	5	4
Infectious eczematoid dermatitis	20	3	11	4	2
Impetigo	13	8	4	1	...
Folliculitis	9	5	2	2	...
Ecthyema	4	1	1	2	...
Sycosis barbae	4	...	2	1	1
Total	75	21	32	15	7
Secondary Infected Dermatoses					
Herpes simplex	6	4	2	...	...
Contact dermatitis	4	1	2	1	...
Atopic dermatitis	3	...	2	1	...
Stasis dermatitis & ulcer	3	...	1	2	...
Tinea pedis	3	1	1	1	...
Tinea capitis	2	...	1	1	...
Seborrheic dermatitis	1	...	...	1	...
Lichen simplex chronicus	1	...	...	1	...
Pediculosis corporis	1	...	...	1	...
Pustular dermatitis-palm	1	...	...	1	...
Total	25	6	9	10	...

DISCUSSION

A comparison of the results of treatment in those subjects treated for primary skin infections involving the uppermost skin structure such as the epidermis and the upper portion of the dermis for example, impetigo, pyoderma, infectious eczematoid dermatitis and folliculitis, to those involving the deeper portion of the dermis, for example, ecthyema or sycosis barbae, revealed the following interesting results. In the former group 49 patients out of a total of 67 patients, or 73.1%, obtained satisfactory results whereas in the latter group only four patients out of a total of eight, or 50%, obtained satisfactory results. This observation suggests that CETAB cream will be most effective in the treatment of those cutaneous infections involving the more superficial structure of the skin.

In the majority of cases treated for secondary infection of preexisting dermatoses, treatment with CETAB cream for 1 week produced sufficient improvement of this complication to allow proper dermatological therapy for the underlying skin condition This was of particular importance where adequate control of the micro-organism was necessary prior to institution of topical steroid therapy.

SUMMARY

The quaternary ammonium compound cetyltrimethylammonium bromide incorporated in a water soluble aromatic mix base as a cream is an effective antimicrobial agent in the treatment of primary and secondary types of skin infections.

Treatment of patients with primary skin infection produced satisfactory results in 71% of the cases treated. Treatment of patients with secondary type skin infection produced satisfactory results in 60% of the cases treated and permitted indicated therapy of underlying dermatoses, in the majority of cases within one week.

CETAB cream is effective against cutaneous pyogenic infections produced either by hemolytic *Staphylococcus aureus coagulase positive* organisms or in combination with hemolytic *Staphylococcus albus*.

The failure of this preparation to produce allergic or irritant reactions, when used in indicated conditions, indicates a low sensitizing and irritant index.

Isolation of Polyporic Acid from *Lopharia papyracea*

By V. JIRAWONGSE, E. RAMSTAD, and J. WOLINSKY

IN RECENT years a mushroom known as Hed Dchig (hed, mushroom; dchig, tummy wood) or Hed Tin Tŭkgae (Tin, foot; tŭkgae, gecko) has come into use in folk medicine in northeastern Thailand as an anthelmintic and is claimed to be effective, especially in the treatment of tapeworm infestations. The mushroom grows on *Careya arborea* Roxb. (*Mytaceae*) and is used as a food. It may be found in local drugstores in Udorn City.

Hed Dchig has been identified as *Lopharia papyracea* (Jungh.) Reid, belonging to the family of *Hydnaceae*.

Received January 29, 1962, from the Departments of Pharmacy and Chemistry, Purdue University, Lafayette, Ind.

Accepted for publication March 7, 1962.

Having available a small supply of the dried mushroom[1] we subjected it to chemical investigation and found it to contain 11.4% of polyporic acid, a compound we found to cause contraction of isolated rabbit ileum suspended in oxygenated Tyrode solution.

EXPERIMENTAL

Isolation.—The leathery tan mushroom was dried over calcium oxide (9.5% moisture), powdered, defatted with petroleum ether by extraction in a

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Identification.—A sample which crystallized from pyridine as orange needles, turning brown on drying, was sublimed *in vacuo* and then recrystallized from chloroform to give shiny purple plates, m.p. 310.5–312°[2] (sealed capillary), $\lambda_{max.}^{EtOH}$ 205, 256, 262, infl. 330 and 465 mμ (ε, 48,000, 43,000, 43,000, 11,600, and 400, respectively), $\lambda_{max.}^{EtOH-NaOH}$ 530 mμ, (ε 200). Literature: m.p. 303–305° (1), $\lambda_{max.}^{dioxane}$ 261, 332 (4.45 and 3.86) (2), $\lambda_{max.}^{pyridine-H_2O}$ 530 mμ (3). The infrared spectrum of this compound was identical with that of an authentic sample of polyporic acid.

Anal.—Calcd. for $C_{18}H_{12}O_4$: C, 73.95; H, 4.14. Found: C, 74.02; H, 4.37.

The diacetate derivative of polyporic acid was prepared using acetic anhydride and pyridine and showed m.p. 212–214° after recrystallization from methylene chloride-ether, $\lambda_{max.}^{EtOH}$ 236, 240 mμ (ε 1,100, 2,350). Literature: m.p. 215° (4); $\lambda_{max.}^{dioxane}$ 240 and 336 mμ (4.33 and 3.78).

The dimethyl ether of polyporic acid was prepared by adding an excess of diazomethane in ether to a suspension of the acid in methylene chloride. The solvents were removed immediately and the residue recrystallized from benzene-petroleum ether to give an orange solid, m.p. 192–194° [reported (5) m.p. 192°].

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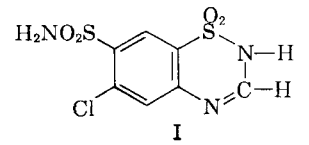
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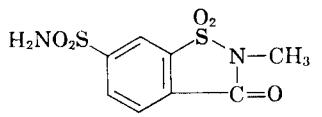
Saccharin Derivatives VI. Synthesis and Diuretic Activity of 2-Methyl-6-sulfamoylsaccharin

By GLENN H. HAMOR

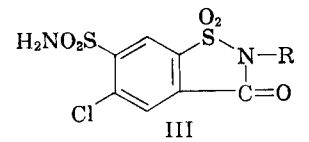
THE APPEARANCE in the literature in 1957 of a report describing the diuretic activity of 6-chloro-7-sulfamoyl-1,2,4-benzothiadiazine-1,1-dioxide (I) (1) led to the preparation of a series of structurally related saccharins. This paper relates the synthesis and result of pharmacological testing of 2-methyl-6-sulfamoylsaccharin (II). Novello, in a recent paper (2), describes the preparation of certain 5-chloro-6-sulfamoylsaccharins (III).



I



II



III

The 2-methyl-6-sulfamoylsaccharin was synthesized by chlorosulfonation of toluene, followed by treatment with 28% ammonia, which gave toluene-2,4-disulfonamide. Oxidation of this compound, succeeded by a Williamson reaction of the resulting 6-sulfamoylsaccharin with methyl iodide, gave the desired 2-methyl-6-sulfamoylsaccharin.

Pharmacological screening in rats has indicated that 2-methyl-6-sulfamoylsaccharin possesses marked diuretic activity (Table I). Testing in the dog for effect on renal clearance of electrolytes and water shows primarily an increase in urine volume accompanied by a slight increase in the excretion of sodium and an increased excretion of potassium.[1]

TABLE I.—DIURETIC EFFECT IN RATS[a]

Dose, mg./Kg. p.o.	Excreted, %	Effect Excreted, %[b]
Control	60[c]	...
5	84	24
15	111	51
30	154	94
60	171	111

[a] Eight rats per group; each group hydrated with 25 ml./Kg. of 0.9% sodium chloride p.o.; length of test was 5 hours. [b] Test minus control. [c] Experience has fixed the control per cent excretion value at 60% for rats. An effect of 22% or greater (82% or more excreted) shows a significant diuretic response.

EXPERIMENTAL[2]

6-Sulfamoylsaccharin.—This compound was prepared by alkaline potassium permanganate oxidation of toluene-2,4-disulfonamide by the method used by Noyes (3) to synthesize 6-nitrosaccharin. The toluene-2,4-disulfonamide was synthesized according to Wynne and Bruce, m.p. 173–174.5° [reported

Received February 12, 1962, from the School of Pharmacy, University of Southern California, Los Angeles.
Accepted for publication March 8, 1962.
The author is grateful to Dr. Glenn E. Ullyot, Smith Kline and French Laboratories, Philadelphia, Pa., for suggestion of the problem.
Presented to the Pharmacy Section Np, A.A.A.S., Denver, Colo., meeting, December 1961.

[1] The author is indebted to Drs. Alfred Maass and Virgil Wiebelhaus, Smith Kline and French Laboratories, Philadelphia, Pa., for pharmacological testing.
[2] Melting points are uncorrected and were determined by the open capillary tube method.

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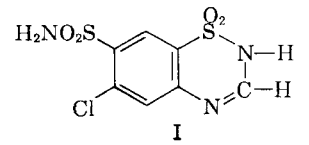
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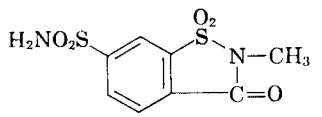
Saccharin Derivatives VI. Synthesis and Diuretic Activity of 2-Methyl-6-sulfamoylsaccharin

By GLENN H. HAMOR

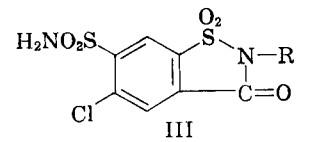
THE APPEARANCE in the literature in 1957 of a report describing the diuretic activity of 6-chloro-7-sulfamoyl-1,2,4-benzothiadiazine-1,1-dioxide (I) (1) led to the preparation of a series of structurally related saccharins. This paper relates the synthesis and result of pharmacological testing of 2-methyl-6-sulfamoylsaccharin (II). Novello, in a recent paper (2), describes the preparation of certain 5-chloro-6-sulfamoylsaccharins (III).



I



II



III

The 2-methyl-6-sulfamoylsaccharin was synthesized by chlorosulfonation of toluene, followed by treatment with 28% ammonia, which gave toluene-2,4-disulfonamide. Oxidation of this compound, succeeded by a Williamson reaction of the resulting 6-sulfamoylsaccharin with methyl iodide, gave the desired 2-methyl-6-sulfamoylsaccharin.

Pharmacological screening in rats has indicated that 2-methyl-6-sulfamoylsaccharin possesses marked diuretic activity (Table I). Testing in the dog for effect on renal clearance of electrolytes and water shows primarily an increase in urine volume accompanied by a slight increase in the excretion of sodium and an increased excretion of potassium.[1]

TABLE I.—DIURETIC EFFECT IN RATS[a]

Dose, mg./Kg. p.o.	Excreted, %	Effect Excreted, %[b]
Control	60[c]	...
5	84	24
15	111	51
30	154	94
60	171	111

[a] Eight rats per group; each group hydrated with 25 ml./Kg. of 0.9% sodium chloride p.o.; length of test was 5 hours. [b] Test minus control. [c] Experience has fixed the control per cent excretion value at 60% for rats. An effect of 22% or greater (82% or more excreted) shows a significant diuretic response.

EXPERIMENTAL[2]

6-Sulfamoylsaccharin.—This compound was prepared by alkaline potassium permanganate oxidation of toluene-2,4-disulfonamide by the method used by Noyes (3) to synthesize 6-nitrosaccharin. The toluene-2,4-disulfonamide was synthesized according to Wynne and Bruce, m.p. 173–174.5° [reported

Received February 12, 1962, from the School of Pharmacy, University of Southern California, Los Angeles.
Accepted for publication March 8, 1962.
The author is grateful to Dr. Glenn E. Ullyot, Smith Kline and French Laboratories, Philadelphia, Pa., for suggestion of the problem.
Presented to the Pharmacy Section Np, A.A.A.S., Denver, Colo., meeting, December 1961.

[1] The author is indebted to Drs. Alfred Maass and Virgil Wiebelhaus, Smith Kline and French Laboratories, Philadelphia, Pa., for pharmacological testing.
[2] Melting points are uncorrected and were determined by the open capillary tube method.

m.p. 190–191°) (4)]. Recrystallization of the 6-sulfamoylsaccharin from acetone gave yields of approximately 40% of white crystalline solid, m.p. 276–278° (decompn.). Herzog has reported the synthesis of 6-sulfamoylsaccharin, but gives no m.p. (5).

Anal.[3]—Calcd. for $C_7H_6N_2O_5S_2$: C, 32.08; H, 2.31. Found: C, 32.02; H, 2.42.

2-Methyl-6-sulfamoylsaccharin.[4]—In a 250-ml. round-bottom flask fitted with a reflux condenser, heating mantle, and magnetic stirrer were placed 1.0 Gm. (0.01 mole) of sodium carbonate, 10 ml. of water, and 5.0 Gm. (0.02 mole) of 6-sulfamoylsaccharin. To this was added 4.26 Gm. (0.03 mole) of methyl iodide in 50 ml. of diethylene glycol monobutyl ether. The solution was refluxed for 3 hours and then poured into approximately 400 ml. ice water. The mixture was let stand overnight in the refrigerator, then filtered to give 3.6 Gm. (68%) of yellow-tan powder, m.p. 230–232°. Recrystallization from ethanol gave a white solid, m.p. 241–242°.

Anal.—Calcd. for $C_8H_8N_2O_5S_2$: C, 34.79; H, 2.9. Found: C, 35.44; H, 3.17.

[3] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.

[4] *Chemical Abstracts* nomenclature: 2-methyl-6-sulfamoyl-1,2-benzisothiazolin-3-one-1,1-dioxide.

REFERENCES

(1) Novello, F. C., and Sprague, J. M., *J. Am. Chem. Soc.*, **72**, 2028(1957).
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(3) Noyes, W. A., *Am. Chem. J.*, **8**, 167(1886).
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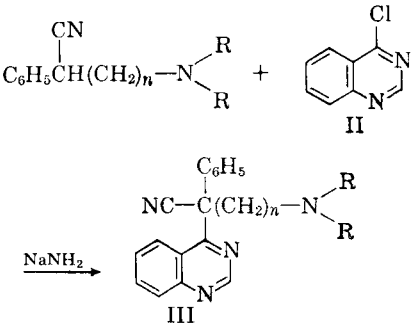
Quinazolines I. ω-Tertiaryamino-α-(4-quinazolyl)-α-phenylalkanenitriles

By RAYMOND N. CASTLE and MASAYUKI ONDA†

Six new ω-tertiaryamino-α-(4-quinazolyl)-α-phenylalkanenitriles have been prepared for pharmacological screening and have been tested for anti-inflammatory activity. Attempts to hydrolyze and decarboxylate these nitriles into the corresponding ω-tertiaryamino-α-(4-quinazolyl)-α-phenylalkanes in acid solution gave none of the desired products. Instead, the only compound isolated was 4-hydroxyquinazoline.

THE PURPOSE of the present work was the synthesis of several ω-tertiaryamino-α-(4-quinazolyl)-α-phenylalkanenitriles for pharmacological screening.

These nitriles were prepared by the methods of Cutler, Surrey, and Cloke (1) with some modification shown in the diagram below. The α-phenyl-ω-tertiaryaminoalkanenitriles used in this work that have not been previously reported in the literature are described in another publication by the present authors (2).



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All six compounds of type III were tested[1] for anti-inflammatory activity in rats. The decrease in intrapleural fluid volume was measured. Compounds 1, 3, 5, and 6 were classed as being active at dose levels of 20 mg./Kg.

EXPERIMENTAL[2]

4-Chloroquinazoline.—The method of Gabriel and Stelzner (4) was modified as follows: a mixture of 3.0 Gm. of 4-hydroxyquinazoline, 6.0 Gm. of phosphorus pentachloride, and 12 ml. of phosphorus oxychloride was heated at 120–130° for 50 minutes. The resulting clear solution was evaporated to dryness *in vacuo* at 80°. Chloroform was added to dissolve most of the solid and the mixture poured on a mixture of crushed ice and concentrated ammonia. The product dissolved in the chloroform layer and this solution was washed with dilute sodium carbonate solution, then with water and dried over anhydrous magnesium sulfate. After evaporation *in vacuo* there was obtained 3.2 Gm. (95%) of a white solid, m.p. 95–97° (literature m.p. 96°) (4).

Preparation of α-(4-Quinazolyl)-α-phenyl-γ-dimethylaminobutyronitrile.—To a solution of 5.4 Gm. of α-phenyl-γ-dimethylaminobutyronitrile in 54 ml. of dry toluene was added 1.3 Gm. of sodium amide and the mixture was refluxed with stirring for 1.5 hours. After cooling, 4.3 Gm. of 4-chloroquinazoline was added and the mixture refluxed and stirred for 3 hours. The mixture was diluted with ether, washed with water and the ether layer

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Accepted for publication March 21, 1962.
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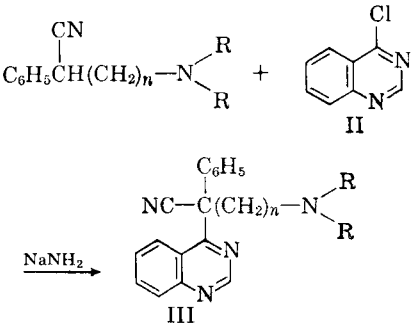
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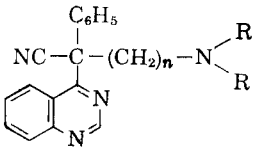
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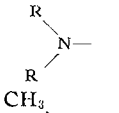
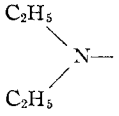
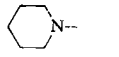
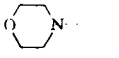
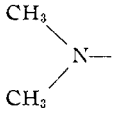
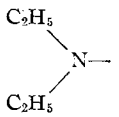
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No.	n	R	B.p., °C.	mm.	Yield, %	Formula	Analyses[a] Calcd. C	Calcd. H	Found C	Found H
1	2		183–186	0.015	85	$C_{20}H_{20}N_4$	75.91	6.37	75.73	6.84
2	2		195–198	0.025	65	$C_{22}H_{24}N_4$	76.71	7.02	77.04	7.19
3	2		200–202	0.025	60	$C_{23}H_{24}N_4$	77.49	6.78	77.16	6.60
4	2		202–205	0.015	57	$C_{22}H_{23}N_4O$	73.71	6.20	73.50	6.75
5	3		182–186	0.005	74	$C_{21}H_{22}N_4$	76.33	6.71	75.82	6.80
6	3		182–185	0.005	71	$C_{23}H_{26}N_4$	77.05	7.31	77.01	7.62

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Convenient Routes to Medicinally Important Heterocycles

By CHARLES S. DAVIS

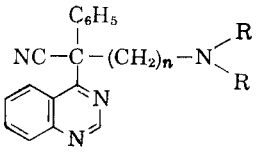
New syntheses of derivatives of benzothiazole, benzoxazole, and benzimidazole are reported.

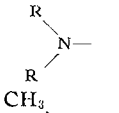
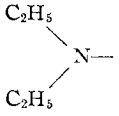
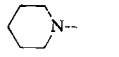
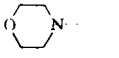
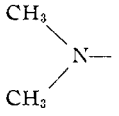
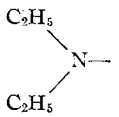
THE benzothiazole, benzoxazole, and benzimidazole rings have long been prominent in pharmacologically active agents (1). Since it is nearly always a problem to the medicinal chemist to prepare specific structures containing these rings, it is felt that additional methods of synthesis may open avenues to new potential drugs.

The reaction between 2-aminobenzenethiol and the phosphorus oxychloride adduct of dimethylformamide was first reported by Davis, *et al.* (2). It was reported that benzothiazole could be prepared in quite good yields by this method. In 1880,

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Convenient Routes to Medicinally Important Heterocycles

By CHARLES S. DAVIS

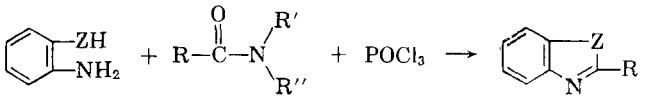
New syntheses of derivatives of benzothiazole, benzoxazole, and benzimidazole are reported.

THE benzothiazole, benzoxazole, and benzimidazole rings have long been prominent in pharmacologically active agents (1). Since it is nearly always a problem to the medicinal chemist to prepare specific structures containing these rings, it is felt that additional methods of synthesis may open avenues to new potential drugs.

The reaction between 2-aminobenzenethiol and the phosphorus oxychloride adduct of dimethylformamide was first reported by Davis, *et al.* (2). It was reported that benzothiazole could be prepared in quite good yields by this method. In 1880,

Received May 11, 1962 from the Research Laboratories of the School of Pharmacy, Purdue University, Lafayette, Ind. Accepted for publication June 11, 1962.

TABLE I.



Z	R	R′	R″	Yield, %	B.p., mm. Hg	Lit.	B.p., mm. Hg
S	CH_3	CH_3	CH_3	82	151 (15)	(4)	151 (15)
S	CH_3	CH_3	H	71	156 (18)	(4)	151 (15)
O	H	CH_3	CH_3	52	185 (747)	(4)	182 (753)
N	H	CH_3	CH_3	50	m.p. 172–173	(5)	m.p. 175
S	—$CH_2CH_2CH_2$—		CH_3	65	144–146 (1.5)[a]		m.p. 104–105[a]

[a] m.p. sulfate. *Anal.*—Calcd. for $C_{11}H_{14}N_2S{\cdot}2H_2SO_4$: C, 32.82; H, 4.57. Found: C, 32.96; H, 4.57.

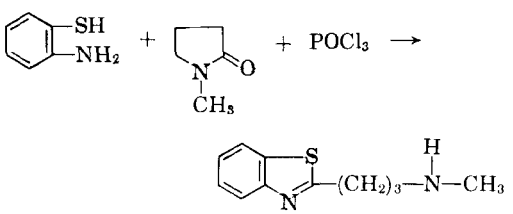
Hofmann (3) reported the preparation of benzothiazole from 2-aminobenzenethiol and formamide. His synthesis required a high temperature and yielded only a small amount of benzothiazole. This note reports convenient syntheses for the derivatives of benzothiazole, benzoxazole, and benzimidazole from the appropriate 2-substituted aniline and a secondary or tertiary amide. A new synthesis for benzimidazole from the acid catalyzed reaction of triethyl orthoformate and *o*-phenylenediamine is also reported.

DISCUSSION

Benzoxazole, benzothiazole, and benzimidazole are formed in good yields when the appropriate substituted aniline is reacted with the phosphorus oxychloride adduct of dimethylformamide. When N-methyl or N,N-dimethylacetamide are reacted with phosphorus oxychloride and 2-aminobenzenethiol, 2-methylbenzothiazole is produced. If acetamide or formamide are allowed to react under the same conditions, no product is formed.

From these experimental results, it is noted that the amide nitrogen must be at least monosubstituted.

To discover the fate of the amide nitrogen in this reaction, it was decided to devise an experiment where the amide nitrogen would remain in the final heterocyclic product. The cyclic amide, N-methylpyrrolidone, was reacted with phosphorus oxychloride and 2-aminobenzenethiol. The product was as expected: 2-(methylaminopropyl)benzothiazole.



EXPERIMENTAL

To a solution of phosphorus oxychloride (0.25 mole) in anhydrous ether (40 ml.) was added dropwise a solution of the appropriate amide (0.25 mole) in anhydrous ether (50 ml.). The addition was carried out at 15°. The amide-phosphorus oxychloride adduct separated as an oily layer or low melting solid and the mixture was allowed to come to room temperature. The adduct was washed with three portions (50 ml.) of anhydrous ether (6).

To the amide-phosphorus oxychloride adduct was added the appropriate *ortho*-substituted aniline (0.125 mole) in anhydrous ether (50 ml.). When the substituted aniline was not sufficiently soluble in ether, the order of addition was reversed. The addition was accomplished with rapid stirring and cooling. After the addition was completed, the ether was evaporated and water (100 ml.) added with cooling. The aqueous solution was extracted with ether and the ethereal solution dried over anhydrous sodium sulfate. The ether was evaporated and the residue distilled; except benzimidazole, which was recrystallized from water. The physical constants of the compounds prepared by this method are listed in Table I.

Benzimidazole *via* the Orthoformate Reaction.—in a 100-ml. round-bottomed flask were placed phenylenediamine (18.4 Gm., 0.17 mole), triethyl orthoformate (37 Gm., 0.25 mole), and 2 drops of concentrated sulfuric acid. The flask was set up for distillation and the ethanol was distilled over a 1-hr. period. After 38 ml. of ethanol was collected, the residue was allowed to cool and solidify. The melting point of the crude material was 162–168°. The solid was recrystallized from water at a melting point of 172–173°; lit. (5) m.p. 175; yield, 17.2 Gm. (88%). This material was identical to that prepared by the phosphorus oxychloride reaction.

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Effect of Gibberellic Acid on *Chenopodium ambrosioides* var. *anthelminticum*

By L. D. KAPOOR and B. K. KAUL

The application of gibberellic acid, also known as GA_3, to *Chenopodium ambrosioides* L. var. *anthelminticum* (L.) A. Gray, induced increases of approximately 25 and 33% in the volatile oil content and yield of fruits. Vegetative growth was also increased. The per cent of ascaridole ($C_{10}H_{16}O_2$) in the treated and control plants was not altered appreciably.

During the course of investigations on the effect of gibberellic acid (GA) with various important aromatic plants, the authors studied the effect of this substance on the growth and production of volatile oil in the chenopodium plant. The per cent of ascaridole in chenopodium oil was also determined. This paper describes the results of our preliminary study with this plant.

EXPERIMENTAL

Young chenopodium seedlings, about 20–30 cm. tall and bearing 15 to 20 leaves, were transplanted during the second week of May 1961 into well-manured beds of six square meters. The first treatment with GA at an average rate of 10 ml. per plant was given on May 28 at the preflowering stage. The same treatment was repeated 1 week later. GA was sprayed in concentrations of 25, 50, 100, and 200 p.p.m. Chenopodium seedlings which were planted in individual clay pots (10 × 12 inches) were simultaneously given GA-spray treatments at fortnightly intervals for 8 weeks in order to determine growth differences.

Observations on Growth.—The plants responded favorably to the various concentrations of GA. Increased internodal elongation was noted in the main shoots of the treated plants. Two weeks after the treatment, the axillary and side shoots appeared in all the treated plants, but in the untreated ones they were found after 4 weeks. After 8 weeks the maximum height (70 cm.) was observed in the plants given a dose of 100 p.p.m. of GA, as compared with 0, 25, 50, and 200 p.p.m. The corresponding figures for these groups were 26, 42, 48, and 68 cm., respectively. Maximum growth responses were observed in plants treated with 100 p.p.m. of GA until the sixth week, but thereafter the differences in length resulting from treatments with the higher concentrations of GA became less pronounced. The leaves were narrower and chlorotic in the treated plants. By the end of June, almost half of the flowers had opened. However, fruit formation was not complete until the end of August. The increased number of nodes and increased elongation of internodes in the treated plants resulted in a large number of axillary shoots bearing more fruit. It was also observed that if the seedlings were sprayed with high concentrations of GA in winter (December to January) the treatment did not initiate a characteristic growth response. However, when the length of day and the temperature were increased, a favorable growth response from GA is controlled by specific temperature and photoperiodic conditions. The optimum period of growth for chenopodium is from April to the end of August.

Fruit and Oil Yield.—The plants were harvested in the first week of September and dried in the shade for a period of 2 weeks. The fruits were then gathered from the dry plant material. The most favorable response on fruit development was induced by treatment with 50 p.p.m. of GA. An increase of about 33% in the dry weights of this group was noted (Table I). The increased amount of fruit was due mainly to the development of a greater number of fruits. This was caused by the formation of additional flowering shoots by the GA treatment. The per cent of volatile oil, determined by steam distillation of the fruits, was found to be up to 25% greater in the treated plants (Fig. 1).

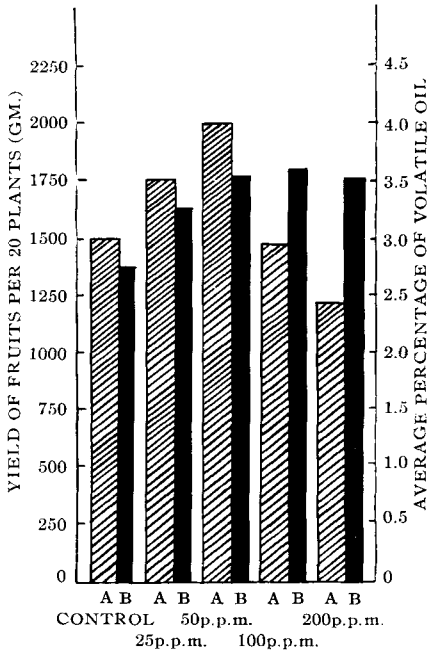


Fig. 1.—Histogram showing the per cent of volatile oil and yield of chenopodium fruits; A = fruit yield, B = % volatile oil.

The oil fractions from the variously treated groups were analyzed for ascaridole ($C_{10}H_{16}O_2$) according to the *Pharmacopoeia of India* (2). The per cent of ascaridole in the oil of treated plants was about the same as in the controls (Table I). It is interesting to note that the ascaridole content for all plants is above the 65% recommended in the *British Pharmacopoeia*.

Received January 15, 1962, from the Regional Research Laboratory, Jammu and Kashmir, India.

Accepted for publication March 5, 1962.

The authors thankfully acknowledge the keen interest of Dr. I. C. Chopra in the work and I. C. I., Ltd., London, for supply of samples of gibberellic acid.

TABLE I.—VOLATILE OIL, ASCARIDOLE, AND FRUIT YIELD IN CHENOPODIUM FOLLOWING TREATMENT WITH GIBBERELLIC ACID

Determination	Treatment with GA, p.p.m. 0	25	50	100	200
Volatile oil in fruits (v/w),[a] %	2.80	3.25	3.45	3.50	3.20
Ascaridole content of oil, %[b]	79.6	79.4	79.0	78.5	78.0
Dry fruit yield, Kg.[c]	1.50	1.75	2.00	1.45	1.25

[a] Mean from 10 determinations. [b] Average from three determinations. [c] Average yield from 20 plants occupying an area of 6 sq. meters.

DISCUSSION

From the foregoing observations it appears that, like *Anethum sp.* (4), the volatile oil of the chenopodium fruits also registers an increase with the application of GA. The fruit yield was also increased, which may be due to longer and more numerous flowering shoots. The increase in percentage of oil in the fruits suggests the possibility that GA may enhance the biosynthesis of essential oils in certain specific organs by either increasing the size and number of the cells which store the essential oil or prolonging the process of synthesis of oil. The ascaridole content in the oil fractions was not, however, altered appreciably by GA treatment but the increase in oil percentage in the chenopodium and anethum is in contrast with the observations on *Mentha spicata* (5) and *M. arvensis* (6) where the percentage of oil was decreased following GA treatment. The difference in behavior by the two categories of aromatic plants is of interest and needs further study.

The observations of Stevens, *et al.* (3), and Kaul and Kapoor (4) suggest that the increase may be due to prolonged biosynthesis of volatile oil and also the increased cell dimensions in the fruits containing the oil.

SUMMARY

1. The plants of *Chenopodium ambrosioides* Linne var. *anthelminticum* responded favorably to the treatment of GA.

2. It induced 25 and 33% increase in the volatile oil and the yield of the fruits, respectively.

3. GA did not appreciably alter the ascaridole content of the oil fractions from the various treatments.

4. The increased yield of fruits was due to increased number of axillary shoots which consequently bore more fruits.

5. The increased oil content in the treated plants may be due to increased cell size of fruits or prolonged biosynthesis of the essential oil.

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Communications

A Modified Method for Preparing Litmus Solution

Sir:

The procedure prescribed by N.F. XI for the preparation of litmus T.S. (1) is tedious and results in a product which is subject to deterioration due to microbial growth. These difficulties may be removed by (*a*) preparing the solution by means of a continuous extractor and (*b*) dehydrating the product by means of freeze-drying. The resulting powder is not subject to microbial growth and may be used to prepare the test solution as needed. The following extraction procedure was used: Place 25 Gm. of powdered litmus in a paper extraction thimble and insert the thimble into a Soxhlet extractor. Add sufficient alcohol to allow for free siphoning into the receiving flask, immerse the flask up to its neck in a steam bath, adjusting the flow of steam to maintain the extracting alcohol at a gentle boil, and continue the extraction for 3 hours. Raise the Soxhlet apparatus so the steam heats enough of the receiving flask to boil only the alcohol in the flask and permit the extraction to continue with the condensor-cooled alcohol for 30 minutes. Draw off all the alcohol in the system and discard. Reassemble the apparatus as it was originally, add sufficient water, arrange for heating with a Bunsen burner, and wash the litmus powder with reflux-cooled water for 3 hours. Discard the water. Remove the extraction thimble with the litmus powder from the extractor, place it in the emptied receiving flask,

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and add 100 ml. of water. Attach a reflux water-cooled condensor to the flask and reflux for 1 hour over a Bunsen burner. Cool the solution and filter. Measure the volume of filtrate collected, and if necessary, add water to make 100 ml. This constitutes the litmus solution.

While the above procedure appears lengthy, it requires for the most part only cursory supervision during the various stages of extraction. The entire extraction, both with alcohol and with water, takes place in the same apparatus using only 75 ml. of alcohol.

The resulting solution can be used as such; but if the volume is more than is required for immediate use, it may be freeze-dried and kept until needed. It is feasible to freeze-dry 100 ml. of the solution in the VirTis freeze-dryer. No doubt other types of dryers are also suitable for this purpose.

The yield of dried residue from 100 ml. of extract is 500 ±2 mg. so that reconstitution can be effected by dissolving the powder in water in the proportion of 5 mg. per ml. Dried material so obtained has not been noted to undergo any change on storage for 12 months.

(1) "The National Formulary," 11th ed., J. B. Lippincott Co., Philadelphia, Pa., 1960, p. 486.

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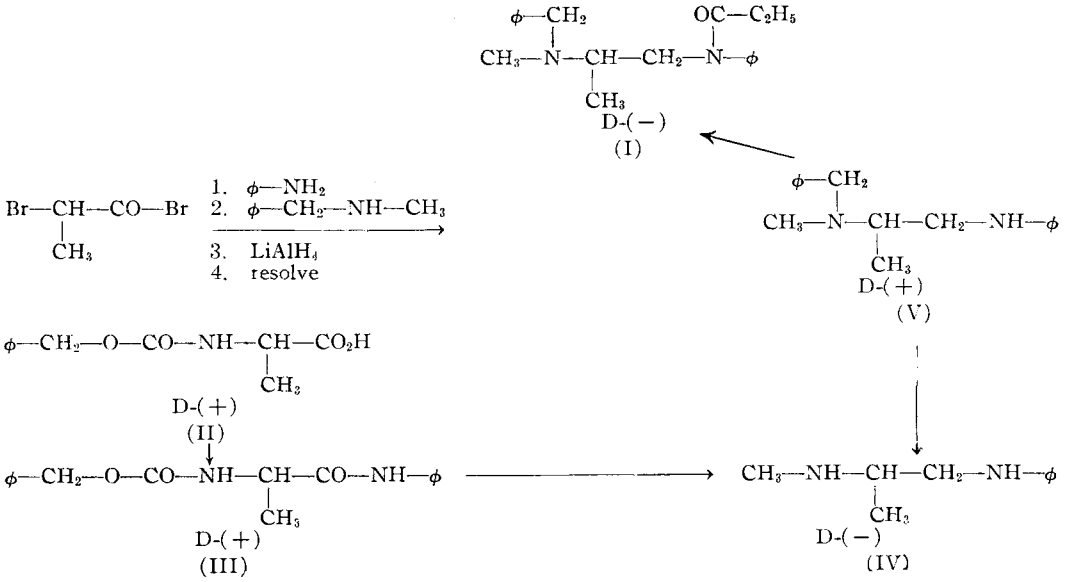
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Received June 19, 1962.
Accepted for publication September 13, 1962.

The Absolute Configuration of (-)-N-[2-(Methylbenzylamino)-propyl]-propionanilide

Sir:

The importance of configurational factors on the activity of various synthetic analgesics has been illustrated by several investigators (1–6). These studies have shown that nearly all the activity resides in only one of the enantiomorphs. Beckett (7–10) has determined that the more active enantiomorphs of methadone-type and thiambutene-type analgesics possess the D-configuration. Though there are only a few classes of analgesics of known absolute configuration, a hypothesis has been proposed which attempts to explain this antipodal specificity on the basis of three point contact with a specific receptor in which only one antipode can fit properly (7). The above findings and speculations have prompted us to determine the absolute configuration of (-)-N-[2-(methylbenzylamino)propyl]-propionanilide (I) (11), a member of a new class of potent analgesics (12). We have related the absolute configuration of I to D-alanine by the following sequence



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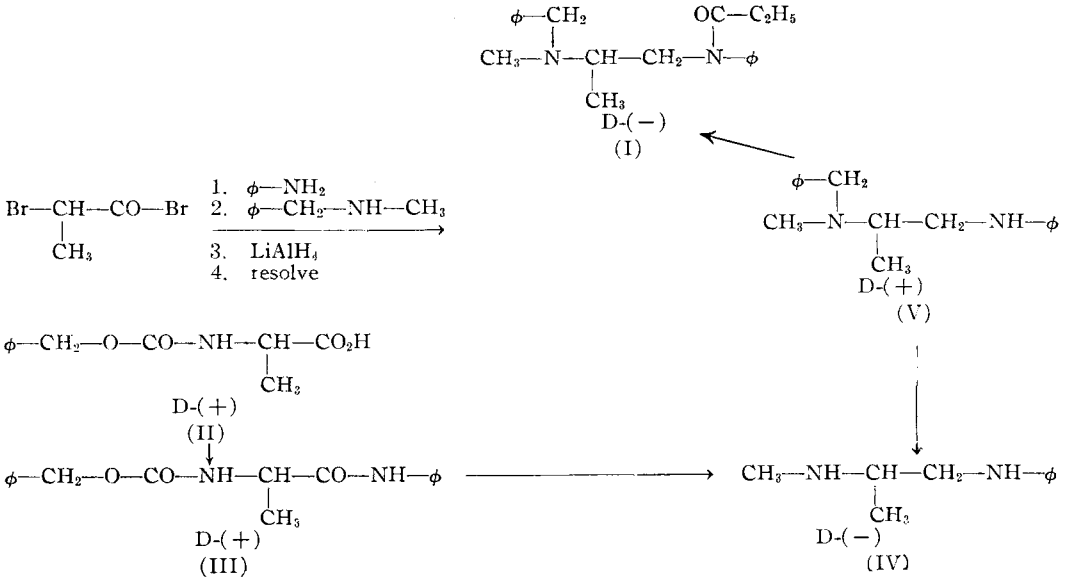
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D-Carbobenzoxyalanine (II) (13) was converted to the corresponding anilide (III), m.p. 162–164°, $[\alpha]_D^{30°}$ +28.4° (c 5% in HOAc), by the action of dicyclohexylcarbodiimide.

Anal.—Calcd. for $C_{17}H_{19}N_2O_3$: C, 67.96; H, 6.38; N, 9.32. Found: C, 68.19; H, 6.13; N, 9.66.

Reduction of III with lithium aluminum hydride in tetrahydrofuran afforded the hygroscopic diamine (IV), m.p. 35–36.5°, b.p. 67–71° (0.1 mm.), $[\alpha]_D^{30°}$ −29.2° (c 5% in ethanol). This compound was analyzed as the dipicrate, m.p. 143–144.5°.

Anal.—Calcd. for $C_{22}H_{22}N_8O_{14}$: C, 42.43; H, 3.56; N, 18.00. Found: C, 42.49; H, 3.60; N, 18.23.

The DL-alkylene diamine (V), m.p. 42–44°, was prepared according to the procedure of Wright, Brabander, and Hardy (11, 12). This compound was previously reported as an oil. Optical resolution of V was accomplished *via* its *d*-bitartrate salt. Five recrystallizations from ethanol afforded a bitartrate, m.p. 101–103°, of high purity. Treatment of the bitartrate with aqueous sodium hydroxide regenerated the resolved diamine (V), m.p. 59–61°, $[\alpha]_D^{27°}$ + 31.2° (c 5% in ethanol). The compound formed a monopicrate, m.p. 168–169°.

Anal.—Calcd. for $C_{23}H_{25}N_5O_7$: C, 57.11; H, 5.21; N, 14.48. Found: C, 56.84; H, 5.33; N, 14.38.

Catalytic hydrogenolysis of the resolved (+)-diamine (V) with palladium-on-carbon catalyst at 40 p.s.i. produced diamine IV, $[\alpha]_D^{27°}$ −29.5°, m.p. 34–35°. The infrared spectrum, gas chromatographic retention time, and dipicrate all were identical with those of diamine IV derived from D-alanine.

Treatment of the D-(+)-diamine (V) with propionic anhydride afforded the D-(−)-enantiomer of I, $[\alpha]_D^{27°}$ −45.7° (c 5% in ethanol), b.p. 152–157° (0.3 mm.). This compound exhibited an infrared spectrum and retention time which is identical with racemic I (11). Wright, Brabander, and Hardy (14) have independently prepared the (+)- and (−)-enantiomers of I. Our physical constants are in agreement with their data.

The analgesic potency ratio between the D- and L-forms will be the subject of a future communication.

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Received August 15, 1962.
Accepted for publication September 5, 1962.
The authors gratefully acknowledge the cooperation of Dr. Robert A. Hardy, Jr. of Lederle Laboratories, in supplying physical data for comparison purposes.
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Studies on the Mechanism of Biotin Action

Sir:

Biotin has been implicated as an essential factor in a wide variety of carboxylation reactions in biosynthetic pathways, but the exact mechanism by which biotin functions remains unknown. For example, in the biosynthesis of purines, it has been suggested (1) that biotin is required in the carboxylation of 5-aminoimidazole ribotide to 5-aminoimidazole 4-carboxylic acid ribotide, which after several further reactions is converted into inosinic acid. Also, in the preliminary steps of fatty acid synthesis, it has been shown that a biotin-containing enzyme is involved in carboxylation of acetyl CoA to malonyl CoA (2). Studies of fatty acid synthesis in certain cell-free extracts have demonstrated that carbon dioxide is activated by a biotin-containing enzyme and that the activated $CO_2 \sim$ biotin-enzyme complex acts as the carboxylating reagent (3). It is unlikely that the carboxyl group of biotin is transferred in this carboxylation reaction since it is known that biotin is covalently bound to protein, probably through an amide linkage to the ϵ-amino group of lysine (4). We have felt that the most logical manner in which carbon dioxide could be activated by biotin is *via* one of the nitrogens of the imidazolidine ring. Recently, evidence has ac-

D-Carbobenzoxyalanine (II) (13) was converted to the corresponding anilide (III), m.p. 162–164°, $[\alpha]_D^{30°}$ +28.4° (c 5% in HOAc), by the action of dicyclohexylcarbodiimide.

Anal.—Calcd. for $C_{17}H_{19}N_2O_3$: C, 67.96; H, 6.38; N, 9.32. Found: C, 68.19; H, 6.13; N, 9.66.

Reduction of III with lithium aluminum hydride in tetrahydrofuran afforded the hygroscopic diamine (IV), m.p. 35–36.5°, b.p. 67–71° (0.1 mm.), $[\alpha]_D^{30°}$ −29.2° (c 5% in ethanol). This compound was analyzed as the dipicrate, m.p. 143–144.5°.

Anal.—Calcd. for $C_{22}H_{22}N_8O_{14}$: C, 42.43; H, 3.56; N, 18.00. Found: C, 42.49; H, 3.60; N, 18.23.

The DL-alkylene diamine (V), m.p. 42–44°, was prepared according to the procedure of Wright, Brabander, and Hardy (11, 12). This compound was previously reported as an oil. Optical resolution of V was accomplished *via* its *d*-bitartrate salt. Five recrystallizations from ethanol afforded a bitartrate, m.p. 101–103°, of high purity. Treatment of the bitartrate with aqueous sodium hydroxide regenerated the resolved diamine (V), m.p. 59–61°, $[\alpha]_D^{27°}$ + 31.2° (c 5% in ethanol). The compound formed a monopicrate, m.p. 168–169°.

Anal.—Calcd. for $C_{23}H_{25}N_5O_7$: C, 57.11; H, 5.21; N, 14.48. Found: C, 56.84; H, 5.33; N, 14.38.

Catalytic hydrogenolysis of the resolved (+)-diamine (V) with palladium-on-carbon catalyst at 40 p.s.i. produced diamine IV, $[\alpha]_D^{27°}$ −29.5°, m.p. 34–35°. The infrared spectrum, gas chromatographic retention time, and dipicrate all were identical with those of diamine IV derived from D-alanine.

Treatment of the D-(+)-diamine (V) with propionic anhydride afforded the D-(−)-enantiomer of I, $[\alpha]_D^{27°}$ −45.7° (c 5% in ethanol), b.p. 152–157° (0.3 mm.). This compound exhibited an infrared spectrum and retention time which is identical with racemic I (11). Wright, Brabander, and Hardy (14) have independently prepared the (+)- and (−)-enantiomers of I. Our physical constants are in agreement with their data.

The analgesic potency ratio between the D- and L-forms will be the subject of a future communication.

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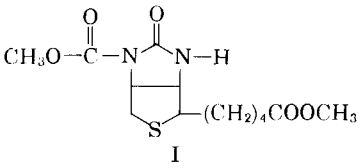
Received August 15, 1962.
Accepted for publication September 5, 1962.
The authors gratefully acknowledge the cooperation of Dr. Robert A. Hardy, Jr. of Lederle Laboratories, in supplying physical data for comparison purposes.
† Fellow, American Foundation for Pharmaceutical Education.

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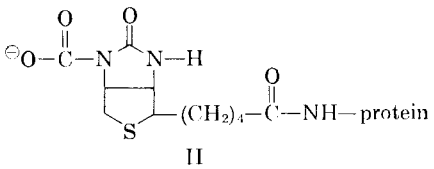
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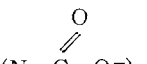
cumulated (3) concerning a biotin-containing enzyme which can utilize free D-biotin as its substrate to give an unstable product which could not be directly characterized but, after treatment with diazomethane, gave the methyl ester of N-carbomethoxybiotin (I) (5). This observation has led to the suggestion (5) that the chemical structure of the $CO_2 \sim$ biotin-enzyme complex may be represented by II.



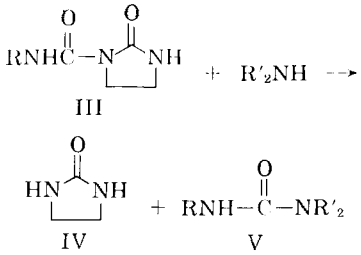
I



II

Compounds with structure  related to II are known to be very unstable and undergo decarboxylation with extreme ease. Therefore, it is probable that the actual structure of the $CO_2 \sim$ biotin-enzyme complex is not one in which the carboxylate anion is present as such but rather one in which the carboxylate anion of biotin has been partially stabilized by reaction with the associated protein. Thus, we visualize these carboxylation reactions as involving the following steps: (*a*) formation of a $CO_2 \sim$ biotin-enzyme complex, (*b*) temporary stabilization of the complex by reaction of the carboxylate anion with a functional group of the associated protein, such as an amino or hydroxyl group, and (*c*) transfer of the carboxyl group to an attacking nucleophilic agent, which would produce the corresponding carboxylic acid and regenerate the biotin-containing enzyme.

In order to gain more information about the mechanism of this reaction, we have undertaken a study of the transfer of a carbonyl group to an attacking nucleophilic reagent using model compounds related to the proposed $CO_2 \sim$ biotin-enzyme complex; *i.e.*, we have studied step (*c*) of the biochemical carboxylation reaction. Because of the instability of compounds with the general structure II, it was necessary to use model compounds which would not undergo spontaneous decarboxylation. The simplest model systems for such a study are the N-substituted derivatives of 2-imidazolidone (III).



III IV V

The model compounds which we chose for our initial study were those in which *R* in III was *p*-methoxyphenyl, phenyl, or *p*-nitrophenyl, and the nucleophilic reagents were morpholine or piperidine.

In a typical experiment, 2-imidazolidone was allowed to react with *p*-methoxyphenyl isocyanate to give a good yield of N-*p*-methoxyphenylcarbamyl-2-imidazolidone (m.p. 175–176°).

Anal.—Calcd. for $C_{11}H_{13}N_3O_3$: C, 56.11; H, 5.52; N, 17.86. Found: C, 56.46; H, 5.28; N, 17.86.

When N-*p* methoxyphenylcarbamyl-2-imidazolidone was allowed to react with morpholine at reflux temperature, the model compound was cleaved to give 2-imidazolidone (IV) and the corresponding urea (V) (m.p. 124–125° in agreement with the literature value (6)).

These reactions represent examples of the transfer of a carbonyl group from model compounds, which are related to the proposed $CO_2 \sim$ biotin-enzyme complex, to a nucleophilic reagent. Although the rate of these reactions is relatively low, experiments with other model compounds and other nucleophilic reagents are in progress in which we hope to observe more facile transfer of the carbonyl group. Nevertheless, the transfer of carbonyl groups in model compounds offers support to the proposed mechanism of action of biotin involving the complex represented by II.

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Received August 3, 1962.
Accepted for publication September 17, 1962.
This investigation was supported by Research Grant RG-9775 from the National Cancer Institute, National Institutes of Health, Public Health Service.
Abstracted in part from research by Parmatma S. Bhargava in partial fulfillment of the M. S. degree requirements.

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1962

JOURNAL OF Pharmaceutical Sciences

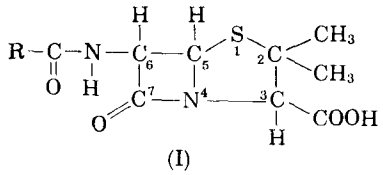
December 1962 volume 51, number 12

Review Article

Pharmaceutics of Penicillin

By M. A. SCHWARTZ and F. H. BUCKWALTER

PROBABLY no group of drugs has received more attention and been more intensively studied than the penicillins (I).



(I)

Their basic structure includes a double ring thiazolidine β-lactam moiety which has been named 6-aminopenicillanic acid (6-APA). The differences in the physical, chemical, and biological properties of the various penicillins are due to their side chains (R in I).

The β-lactam ring is quite labile in all the penicillins known at present. Differences in stability of the several penicillins are determined by the side chains, the structural characteristics of which confer varying degrees of protection against degradation.

Before the isolation of 6-APA from fermentation broths by a group at the Beecham Laboratories in England in 1958 (1), complete penicillins could be made commercially only by fermentation, and variations in the side chain were made by the addition of appropriate precursors to the fermentation broth. This severely limited the number and types of penicillins which could be produced. Since 1958, however, penicillins have achieved a revived importance in chemotherapy because now, theoretically any side chain may be linked by chemical means with the 6-APA nucleus to form a new penicillin. In addition, other workers (2–4*a*) have found enzymes which will selectively hydrolyze penicillin at the amide linkage to produce 6-APA.

Because of this revived interest in penicillins, their stability and incompatibilities with other medicinals have become of major importance to pharmacists in every area of the profession. It is the purpose of this paper to review and correlate all the available data on penicillin stability. Penicillin G (I, R = $C_6H_5CH_2$—), on which most of the work has been done, will be discussed first, and then the newer penicillins compared with it.

PENICILLIN G

Routes of Degradation.—Figure 1 depicts the major routes of degradation of penicillin G of importance in pharmaceuticals. In neutral or alkaline medium it is hydrolyzed at the β-lactam to penicilloic acid (II) (5). The reaction at constant temperature and pH is first order with respect to penicillin and is directly proportional to hydroxyl ion concentration (6). Benzylpenicillin is much more reactive toward alkali than simpler β-lactams, and this has been attributed to facilitation of hydrolysis by the sulfur bonded to the β-lactam ring and/or fusion of the β-lactam ring with a five-membered ring (7). The mechanism of this reaction in neutral solution is not completely understood, but several workers (8, 9) have obtained evidence from ultraviolet absorption data that it proceeds through penicillenic acid (III). This compound has been isolated and shown to have a maximum

Received from the Research Division, Bristol Laboratories, Syracuse, N. Y.

Presented, in part, to the Pharmaceutical Technology Section, A.PH.A., Las Vegas meeting, March 1962.

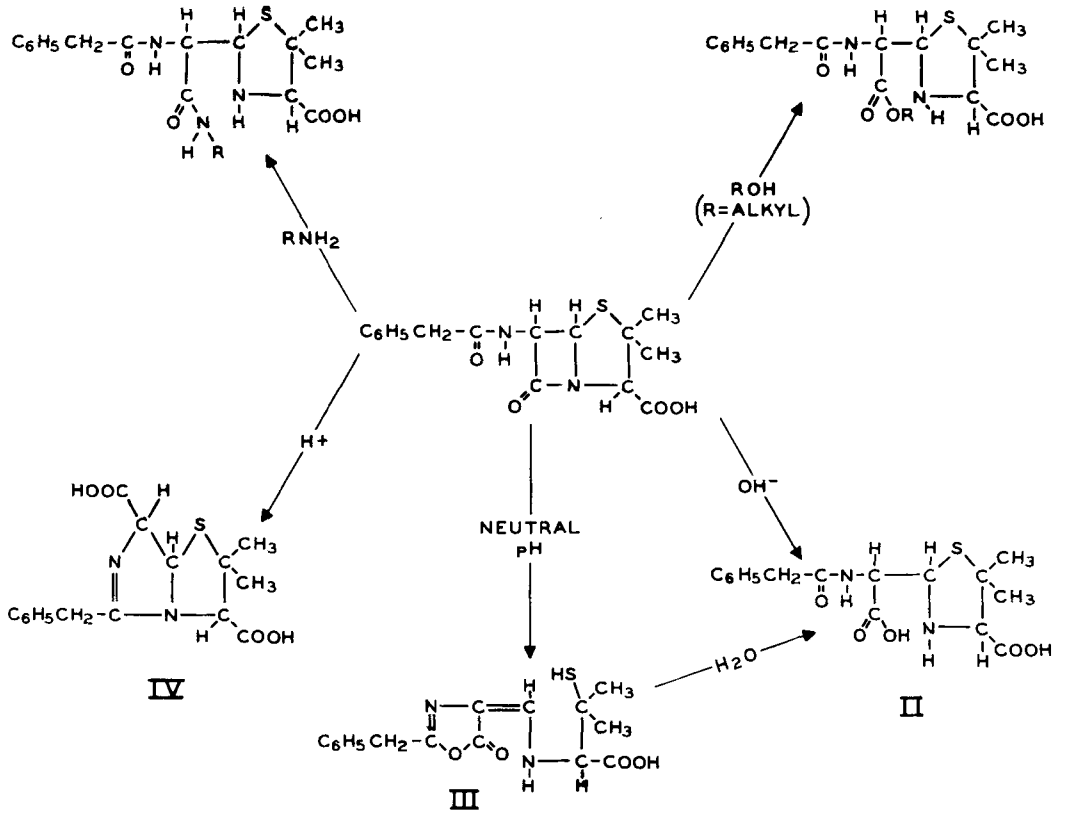


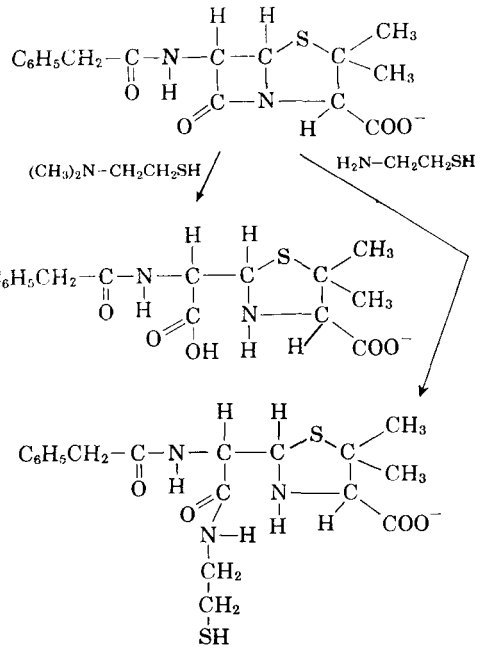
Fig. 1.—Major degradative reactions of penicillin.

in its ultraviolet absorption spectrum at 322 mμ (10). During hydrolysis of penicillin at pH 7.5 the absorption at 322 mμ increases, indicating the presence of this compound. It is rapidly converted to penicilloic acid with half-life, at pH 7.5 and 37°, of 6.5 minutes (10). The overall half-life of penicillin G under these conditions is about 60 hours. In strongly alkaline solution, penicillin G is probably hydrolyzed directly to penicilloic acid. In strong acid solution, penicillin is rearranged to penillic acid (IV) (5). At constant temperature and pH, this reaction is also first order (11) and depends on hydrogen ion concentration (12–14). This dependence is not linear due to the ionization of penicillin which has a pKa about 2.8 and, therefore, exists in two forms, free acid and ion, in the pH range from about 1.5 to 4.5. Each of these species is inactivated at a different rate, and this produces the curvature in the pH profile of Fig. 2. Similar pH dependence, although at different rates, has recently been demonstrated for phenethicillin (15) (I, R = C_6H_5OCH(CH$_3$)—) and this is also shown. Penicillins F, K, and X, all of which were made by fermentation, are also inactivated at different rates (16), but are not important therapeutically.

From the pH profile it may be observed that the pH of minimum degradation is about 6.5. This naturally is quite important in formulation of pharmaceuticals.

Penicillin reacts fairly rapidly with certain alcohols and amines to form the esters and amides, respectively, of penicilloic acid (5).

The reaction of penicillin with cysteine and some related mercaptoamines has been investigated from a mechanistic standpoint (17). With primary mercaptoamines, the products were found to be N-penicilloylamides, whereas with tertiary mercaptoamines the product was free penicilloic acid. From the pH dependence of the reaction it was concluded that the active species was a mercaptide ion and the rate-limiting step was formation of a semimercaptol intermediate. In the case of the primary mercaptoamines, this intermediate rearranged to the amide. With the tertiary mercaptoamine, amide formation was impossible and the product was penicilloic acid.



Some other reactions of penicillin of pharmaceutical interest will be discussed below in the section on imcompatibilities.

One other route of degradation which is more important therapeutically, but which deserves mention here, is the hydrolysis of penicillin by penicillinase. This enzyme is formed by certain microorganisms, notably certain strains of staphylococci, and rapidly catalyzes the hydrolysis of penicillin to penicilloic acid. The reaction is known to follow the usual Michaelis–Menten kinetics (18, 19). Certain compounds have been shown to inhibit penicillinase *in vitro* (20–24) but none of these has been used therapeutically.

STABILITY OF PENICILLIN IN DOSAGE FORMS

Solid Dosage Forms.—The most critical factor governing stability of penicillin in the solid state is moisture content. In one study of crystalline sodium penicillin G (25) it was shown that when the moisture content in vials was above 4%, significant loss of potency (40–80%) occurred after 6 months at 25°. This effect was much less pronounced at refrigerator temperature. Thus, the hygroscopic capacity of the powder and the type of closure on the vial are extremely important (26).

In the early days of penicillin amorphous forms of the salt were often used. It was generally found that the crystalline forms were much more stable than the amorphous, although this could not be correlated with moisture content (27). It was also found that the calcium salt was less hygroscopic than the sodium salt (28). Nevertheless, calcium penicillin has not seen wide usage.

A study of many commercial lots of crystalline sodium and potassium penicillin in vials showed that there were only slight losses (1.5–2%) after 3 years storage at room temperature (29). Thermolability of sodium penicillin has been followed by several workers (30, 31) and it was found that the antibiotic could be heated at 100° for 4 days with no more than 10% loss in activity. At temperatures over 140° however, loss was quite rapid.

Relatively insoluble amine salts of penicillin, such as the procaine and dibenzylethylenediamine salts, are also stable as dry powders for periods of 3 years or more at room temperature (32), as are combinations of procaine and potassium

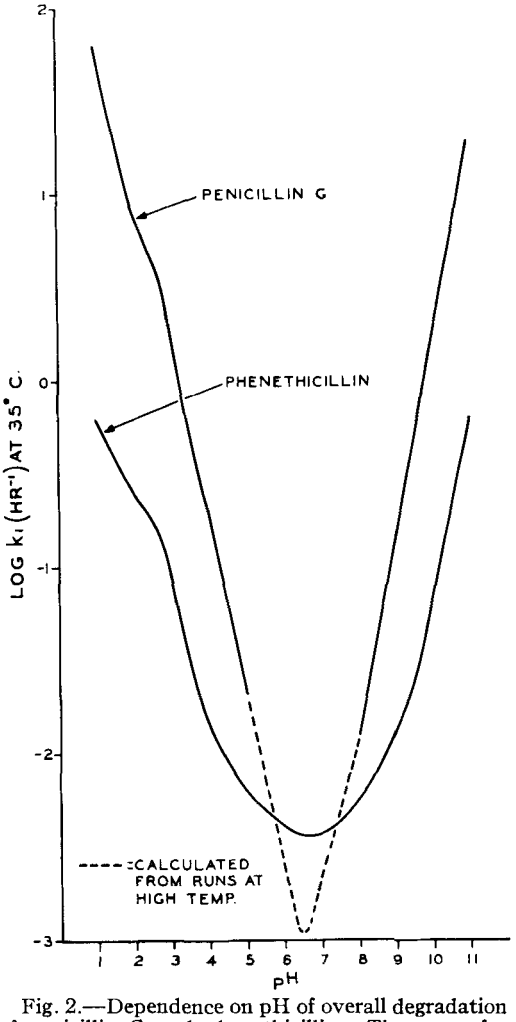


Fig. 2.—Dependence on pH of overall degradation of penicillin G and phenethicillin. The curve for penicillin G was taken from ref. 16.

penicillins (33). Buffered penicillin powder, marketed for oral use after reconstitution with water, and combined with flavors, dyes, and sweeteners, was found to exhibit virtually no loss in potency after storage at room temperature for 4 years (34).

The stability of penicillin tablets is also dependent on moisture content. An early report (35) showed that tablets packaged with silica gel were much more stable than those packaged without the moisture absorber. Later it was shown that buffered penicillin tablets (36) and soluble tablets in hermetically sealed aluminum foil (37) were stable for at least 3 years, as were troches of potassium and procaine penicillin (38, 39). It has also been stated that sugar coating improves the stability of penicillin in tablets (40).

Liquid Dosage Forms.—As noted in referring to the kinetic studies of the degradation of penicillin in aqueous solution, the antibiotic is destroyed fairly rapidly and this reaction is catalyzed by both acid and base. It has been noted also that the reaction in neutral solution produces acid (penicilloic). From a glance at the titration curve of penicillin in Fig. 3 it can be seen that a small amount of acid will lower pH very rapidly. Thus, in a solution of penicillin in plain water, one may expect the rate of hydrolysis to increase as the initial reaction lowers pH to a point where the acid-catalyzed reaction becomes appreciable. It was recognized quite early that it was necessary to buffer solutions of penicillin near neutral pH in order to obtain optimum stability (41, 42). A controversy arose as to whether phosphate or citrate was the better buffer system. Hahn (43) claimed that citrate stabilized penicillin to a greater extent than would be indicated by its buffering action alone. He compared stability in citrate and phosphate buffer of the same pH and found that the solution in citrate buffer was more stable. Pulvertaft and Yudkin (44) claimed that phosphate also stabilized penicillin beyond its buffer effect yet, at the same time, showed that there existed an optimum concentration of phosphate for a particular concentration of penicillin. If phosphate were exerting some stabilizing effect, one would expect higher concentrations to stabilize even more so. The fact that higher concentrations caused less stability suggests that phosphate buffer had some catalytic effect on hydrolysis of penicillin. Pratt (45) found that phosphate buffers of a particular concentration stabilized penicillin better than lower concentrations, but found no difference in half-life when concentrations above his "optimum" were used. This indicates that the lower concentrations were simply of too low a buffer capacity for the concentration of penicillin used. Thomas (46) claimed that phosphate, citrate, and acetate buffers all stabilized penicillin. On the other hand, Macek, *et al.* (47), presented evidence which showed that the stabilizing effect of phosphates and citrates depends on their buffering action and not on any specific ion effect. Other workers (48, 49) have shown that increasing concentrations of penicillin require increasing concentrations of phosphates to maintain optimum stability. It thus appears that the action of buffers in stabilizing penicillin is strictly related to their ability to maintain pH. It should be noted here that the penicillin available at the time most of these studies were being done was of doubtful purity and the impurities may have been such as to cause some error. For example, buffer materials from the fermentation medium were often carried over to the final penicillin powder.

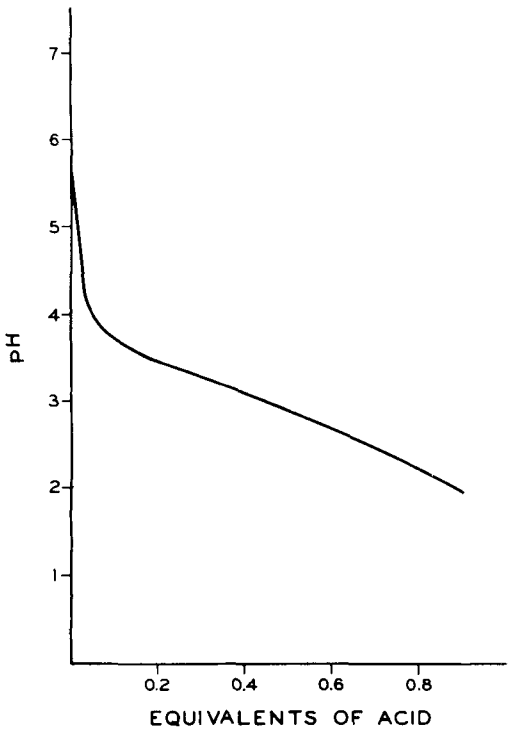
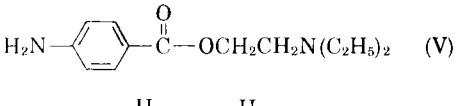


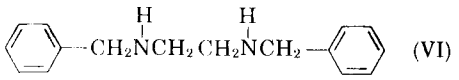
Fig. 3.—Titration curve of penicillin (potassium salt titrated with acid).

From what has already been said it is obvious that a solution of penicillin cannot, with present techniques, be made stable for more than about 2 weeks even at refrigerator temperatures. Partly for this reason, many sparingly soluble amine salts of penicillin were prepared. In these,

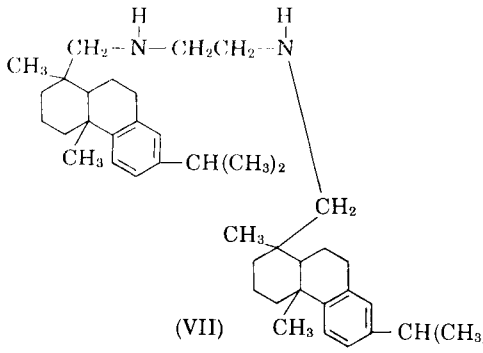
the protonated amine replaces the potassium or sodium of the soluble salt. Of these only a few have been marketed, *e.g.*, procaine (V), N,N′-dibenzylethylenediamine (VI), and N,N′-bis-(dehydroabietyl) ethylenediamine (VII). It was



(V)



(VI)



(VII)

found that suspensions of these salts in aqueous vehicles could be made stable for a year or more, allowing marketing of a "ready-made" penicillin product.

The stability of these salts in aqueous suspension is based mainly on their solubility and the rate of degradation of material in solution. A theoretical treatment of the solubility of these salts has been presented by Brunner (50, 51). He has derived equations by which one may calculate solubility as a function of pH, and pH of minimum solubility. These are based on mass-action law, a knowledge of the ionization constants of the amine and penicillin, and the solubility of the salt in pure water. The rate of loss of penicillin from a suspension in which the two phases are kept at equilibrium at constant temperature and pH will be pseudo zero order where the rate constant (k_0) will be the product of the first-order rate constant (k_1) for the material in solution, and the saturation concentration of penicillin under these conditions

$$\frac{-d(\text{Pen})}{dt} = k_0; \quad k_0 = k_1(\text{Pen})_{\text{sat.}}$$

Since the salt in solution is dissociated, or at least partly dissociated, some suppression of solubility may be achieved by the common ion effect. This has been demonstrated by Swintosky, *et al.* (52), with procaine penicillin by addition of procaine hydrochloride. These workers further reduced the solubility of the penicillin salt by the addition of salts and sorbitol to the vehicle. The rates of degradation of saturated solutions of procaine penicillin in this vehicle at several temperatures, as well as solubility as a function of temperature, were also measured (53). From these data predictions of long range stability of the product were made. These agreed fairly well with experimental values despite many assumptions made in the calculations.

While amine salts offered a means of stabilizing liquid penicillin preparations, generally their low solubility led to reduced penicillin serum levels after oral administration, as compared with those observed after oral administration of a soluble penicillin salt. It was demonstrated (54) that a stable oral liquid preparation of potassium penicillin could be made by suspending the salt in an edible, modified coconut oil. This preparation provided penicillin serum levels similar to those achieved with previously available preparations of the potassium salt.

Slawinska (55) showed that ultrasonic waves had a deleterious effect on stability of penicillin in aqueous solution, but not in paraffin suspension.

Stability in Ointments.—As is the case with powders and tablets, the stability of penicillin in ointment bases depends mostly on water content (56–58). In anhydrous bases the penicillin is relatively stable (59), whereas in emulsion bases the opposite is true. In Carbowax bases penicillin is also quite unstable (60, 61). Peroxides also appear to have a deleterious effect on penicillin stability. It was found that penicillin ointments made with peroxide-bleached bases lose about 30% potency in 7 months (62). If the peroxide value of petrolatum is kept below 0.06%, stability will be much greater (63). Procaine penicillin ointment made with a base consisting of peanut oil, beeswax, and petrolatum was stable for 4 years at room temperature (64).

INCOMPATIBILITIES OF PENICILLIN

Table I summarizes the reported incompatibilities of penicillin together with the appropriate literature references. As previously noted, penicillin is incompatible with acid and alkali and, in one sense, even with water. Any material which, when mixed with penicillin in solution, will change the pH away from the neutral region will be incompatible.

Generally alcohols, glycols, polyglycols, glycerin, and some sugars appear to react with

TABLE I.—INCOMPATIBILITIES OF PENICILLIN G

Type Compound	Reference
Oxidized cellulose	65
Chlorocresol	66
Glycerin and glycols	66–71
Heavy metals	72–76
Amines	77–80
Resorcinol	81
Zinc oxide	81, 82
Vitamin B_1	81
Procaine	81
Ephedrine	81, 83, 84
Iodine and iodides	81
Alcohols	71, 81, 85
Oxidizing agents	76, 79
Rubber tubing	86, 87
Thiols	88–92
Sugars	93, 94
Acids	82, 95
Aminoacridine hydrochloride	71
Thimerosal	71
Certain flavors	96

penicillin, probably by esterification of the potential acid group of the β-lactam, thereby forming biologically inactive penicilloates. Thiols act in a similar manner, although thiosulfate and metabisulfite have no deleterious effect (97). It has been reported, however, that penicillin is rapidly inactivated by sulfite and bisulfite (98). There was no sulfite found in the product and 82% of the original sulfite was recovered, indicating that some catalytic pathway is involved. Certain types of rubber tubing inactivate penicillin due to the presence of mercaptans from the vulcanization process (86, 87). Traces of heavy metals catalyze the hydrolysis and alcoholysis of penicillin. While the mechanism of this catalysis is not understood, it appears to involve the sulfur of the thiazolidine ring. Oxidizing agents also react with penicillin but the mechanism also is not known. The loss in activity might be caused by opening of the thiazolidine ring and oxidation of the sulfur to a sulfonic acid (penicillaminic acid) (76).

Amines may be incompatible with penicillin in two ways: precipitation of a salt if the latter is insoluble and/or reaction to form a penicilloamide.

STABILIZATION OF PENICILLIN

From the picture presented thus far, certain generalizations may be made concerning means of stabilization of penicillin in pharmaceutical preparations.

With solid dosage forms and ointments, the presence of water should be avoided to the maximum extent possible. This includes processing of materials during manufacture. It has been shown that dehydrating agents improve the stability of ointments containing up to 0.1% water (99).

The most important single factor in liquid formulations is pH control. Products made for solution will have maximum stability when pH is kept at about 6.5 by a buffer of sufficient capacity to maintain that pH throughout the useful life of the product.

With suspensions of amine salts, both solubility and rate of degradation are functions of pH, as shown in Fig. 4. The pH of optimum stability will be that at which the product of solubility and rate is a minimum. In addition, anything which reduces the solubility of the salt will aid stability, *e.g.*, addition of a soluble salt of the amine, salting out, etc. As a corollary, any substance which tends to solubilize the salt should be avoided in formulation. Since penicillin is known to react with certain amines, those chosen for preparation of insoluble salts should be unreactive toward the drug.

Claims have been made that several compounds "stabilize" penicillin in solution. Hobbs, for example, claimed that hexamine improved the stability of sodium penicillin G in solution (100). To the authors' knowledge this has not been refuted. When hexamine was added to a suspension of procaine penicillin, however, stability was decreased. This was due to some solubilization of the salt by the hexamine (101).

A German patent (102) claims the stabilization of penicillin solutions with soluble salts of 8-chlorotheophylline. The inventors claim a complex is formed in which the β-lactam is protected from hydrolysis. The preparation of this complex is given in a recent publication

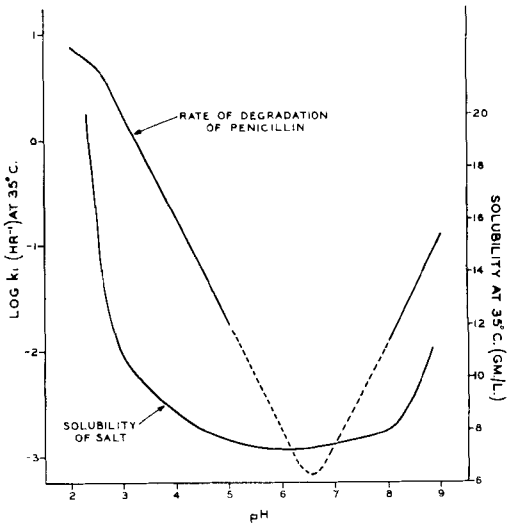


Fig. 4.—Solubility of procaine penicillin G and rate of degradation of penicillin G as functions of pH.

(103). Since the 8-chlorotheophylline is acidic, it is possible that this may be another case of a buffer effect causing stabilization. 8-Chlorotheophylline was ineffective as a stabilizer for phenethicillin in buffered solutions at pH 6.5 (104).

Aminopyrine (105) and sodium hexametaphosphate (76) also appeared to stabilize penicillin in solution, but again the comparisons were made with unbuffered solutions.

Paolini, *et al.* (106), have presented evidence to show that sodium sulfamethoxypyridazine "exerts a protective action against degradation of penicillin G sodium in aqueous solution at pH greater than 7." Their experiments however, were conducted in such a manner that pH dropped during each run, and this could have a significant effect on rate of hydrolysis. This is especially true above pH 7.5 where the rate becomes very highly pH dependent. Further work will be required to establish definitely whatever utility sulfonamides may have as stabilizers of penicillin.

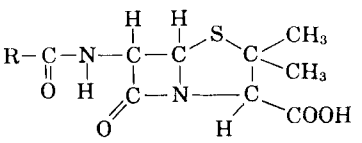
NEWER PENICILLINS

This section will compare the stability of the newer penicillins with penicillin G. For purposes of convenience penicillin V is included in this group although it should not be categorized with the synthetic penicillins. The chemical structures and nomenclature of these compounds are shown in Table II.

Acid Stability.—From a therapeutic standpoint, stability of penicillins to acid is quite important since it may be a limiting factor in oral effectiveness of the drugs. Table III gives the approximate half-life, at pH 1.3 and 35°, of several penicillins for which data are available. The most stable of these is ampicillin which has been recently marketed in Great Britain, the half-life being about 200 times that of penicillin G. This has been attributed to the electron attraction of the positively charged amino group which might be expected to hinder the electron displacements initiating rearrangement to the penillic acid (111). The authors also found a qualitative correlation between pKa of the side chain acids and acid stability of the penicillins. This should be useful in predicting acid stability of new penicillins, at least within a given structural group.

Stability in Pharmaceuticals.—As was the case with penicillin G, none of the newer penicillins is sufficiently stable in aqueous solution to

TABLE II.—NEWER PENICILLINS



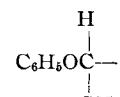
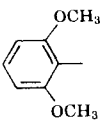
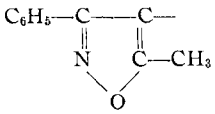
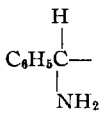
R	Nonproprietary Name	Chemical Name	Trade Name
$C_6H_5OCH_2$—	Penicillin V	Phenoxymethyl-penicillin	V-Cillin, Lilly Pen-Vee, Wyeth
	Phenethicillin	α-Phenoxyethyl-penicillin (107)	Syncillin, Bristol Darcil, Wyeth Chemipen, Squibb Alpen, Schering Maxipen, Pfizer Rocillin, Rowell Semopen, Massengill Dramcillin-S, White
	Methicillin	2,6-Dimethoxy-phenylpenicillin (108)	Staphcillin, Bristol Dimocillin, Squibb
	Oxacillin	3-Phenyl-5-methyl-4-isoxazolylpenicillin (109)	Prostaphlin, Bristol Resistopen, Squibb
	Ampicillin	α-Aminobenzyl-penicillin (110)	Penbritin, Beecham Laboratories (Great Britian)

TABLE III.—ACID STABILITY OF PENICILLINS

Penicillin	Half-life, min.[a]	Reference
"G"	3.5	111
"V"	160	111
Phenethicillin	68[b]	15
Methicillin	2.3	112
α-Methoxybenzyl	77	111
α-Chlorobenzyl	300	111
Ampicillin	660	111
Oxacillin	160	112

[a] pH 1.3, 35°, in 50% alcohol. [b] In aqueous solution (no alcohol present).

be marketed as such. The rate of degradation of phenethicillin in aqueous solution as a function of pH and temperature was investigated and found to follow the same general pattern as penicillin G, although the rates were slower (15). The pH of maximum stability was 6.5 and secondary phosphate ion was found to catalyze the loss of activity. Based on these data, a powder for reconstitution by the pharmacist at the time of dispensing was formulated. This lost only about 1% of its penicillin potency when reconstituted and stored for 2 weeks at refrigerator temperature (113). Phenethicillin in solution was studied in combination with 77 common liquid prescription preparations, most of which had no effect on stability for 3 days at 4° (113). Those which had the greatest effect were the products which changed the pH outside the range for maximum stability.

Methicillin is relatively unstable in aqueous solution. It has been reported that solutions kept at room temperature should be used within 12 hours of preparation and those stored at 4° within 24 hours (114). Work done in our own laboratories indicates that methicillin buffered with sodium citrate has much greater stability in solution.

Oxacillin is stable for about 24 hours at room temperature in aqueous solution buffered with sodium citrate (115).

Ampicillin, at neutral pH and room temperature, maintained 80% of its activity for 1 week (116). Whereas one would suspect that the other newer penicillins would show a dependence of rate of loss of activity on pH similar to that of penicillin G, one might also speculate that ampicillin might be different because of its zwitterionic character at almost neutral pH. From the reports available to date it appears to be the most stable penicillin available, in both acid and neutral solution.

Stability to Penicillinase.—It has been shown that the Michaelis constants for the catalysis of hydrolysis of these penicillins by staphylococcal penicillinase is in the order expected from previous experience with these drugs in treatment of infections caused by staphylococci resistant to penicillin G (117). That is, the greatest affinity for the enzyme is shown by penicillin G and the least by methicillin and oxacillin, the others being intermediate. Ampicillin is rapidly inactivated by penicillinase (112) but kinetic data are not available.

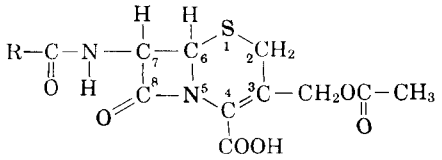
FUTURE PENICILLINS

Now that the means of making almost any penicillin is available, one may speculate on what the future may bring in this field. This might be done in terms of the properties desired in the ideal penicillin: (*a*) it should be active against a broad spectrum of microorganisms, including possibly fungi and viruses; (*b*) it should be sufficiently acid-stable so that it will not be destroyed in the stomach; (*c*) it should be rapidly and completely absorbed from all sites in the gastrointestinal tract, and slowly excreted; (*d*) it should not be strongly bound by serum protein; (*e*) it should be stable in aqueous solution; (*f*) it should be resistant to attack by penicillinase and/or other enzymes; (*g*) it should be nonallergenic; (*h*) it should be nontoxic.

Each of the newer penicillins has only two or three of these properties and, indeed, it may turn out to be impossible to make a single penicillin with all of them. Nevertheless, these criteria point the way for future research.

With regard to stability, it may be possible to make penicillins which will be stable in solution. In order to design such molecules further insight into the mechanisms of inactivation and the effects of structural changes upon these should be gained.

One group of compounds similar to the penicillins, but appearing to have much greater stability, are the cephalosporins. These are naturally occurring compounds which are derivatives of 7-aminocephalosporanic acid (VIII).



(VIII)

One of these, cephalosporin C

(R = H_3N^+—CH(—OOC)—$CH_2CH_2CH_2$—)

was found by Abraham and Newton (118) to inhibit competitively the hydrolysis of penicillin G by

penicillinase. These authors also isolated 7-aminocephalosporanic acid in very small quantities and acylated it with phenylacetyl chloride to obtain the derivative corresponding to penicillin G (119). This compound was several hundred times more active than cephalosporin C and yet resistant to hydrolysis by penicillinase. Apparently difficulties have been encountered which have prevented commercial production of these compounds thus far.

One other area which requires further exploration is the mechanism of action of penicillinase and the structural features of its active sites. This knowledge may enable us to design inhibitors of the enzyme.

SUMMARY

A survey of knowledge to date on stability of penicillins has been presented, including acid stability, stability in pharmaceutical preparations, and stability to the enzyme penicillinase. Incompatibilities and possible "stabilizers" were also discussed.

The side chain structure of the various penicillins endows each with different stability characteristics, although none available at present is sufficiently stable to allow marketing in solution.

Despite the great improvements in penicillins which have been made in the past 3 years, there is still much room for further work. With current ability to make structural modifications relatively easily, it is expected that even better penicillins will be seen in the future.

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Research Articles

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By WINTHROP E. LANGE, ALFRED G. BARNET†, and WILLIAM O. FOYE

Several commercial hematinic preparations have been tested for their ability to liberate ionic iron at different conditions of pH and in the presence of simulated gastric and intestinal fluids. A chromatographic method was employed in which both Fe(II) and Fe(III) ions were measured by means of absorbance determinations. It was concluded that chelated, or complexed, iron is carried through the gastrointestinal tract with less loss and lower toxicity than is ionic iron.

Some confusion exists regarding the requisites for the state of iron necessary for therapeutic use as a hematinic. Brading, *et al.* (1), for instance, have reported that more Fe(III) than Fe(II) was absorbed by rats fed inorganic Fe^{59}, and that the distribution in the tissues did not depend on either dose or valency of iron. Hartwig, *et al.* (2), also found that $Fe^{59}Cl_3$ was incorporated into new erythrocytes to a significantly greater degree than was Fe^{59}(II) citrate. Elsewhere it has been stated that only ferrous iron can be absorbed and that the ferric form must first be reduced before it can enter the gastrointestinal mucosa (3).

The advantages of chelated *vs.* inorganic iron for therapeutic use also appear to be in question. Franklin, *et al.* (4), claimed that chelation of iron minimized its toxicity and did not impair its hemopoietic response in humans. A difference in effectiveness of iron chelate preparations has been noted (5), however, which is evidently due to relative ease of liberation of ionic iron from iron chelates. Injectable ferric ammonium citrate, for instance, gave low hemoglobin levels and erythrocyte counts in weanling pigs in comparison with injectable iron-dextran, oral iron in

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Accepted for publication May 25, 1962.
Abstracted from a thesis submitted by A. G. Barnet as a requirement for the degree of Master of Science, 1961.
Presented to the Scientific Section, A.Ph.A., Las Vegas meeting, March 1962.
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paste form, and ferrous sulfate. Only the ferrous sulfate maintained normal blood values.

It is well known that ferric chelates show much more stability than ferrous in regard to liberation of ionic iron, since Fe(III) falls well above Fe(II) in the Mellor-Maley and Irving-Williams series. Also, in regard to oxidation-reduction potentials, it is known that when a ligand is anionic, the higher valence state of the metal is favored (6). Furthermore, ferric iron in many of its chelates is more difficult to reduce than inorganic ferric ion (6, 7).

The foregoing facts lead obviously to questions regarding the availability of iron in hematinic preparations where chelating agents are employed, the possibility, or necessity, of reduction of Fe(III) to Fe(II), and the competition of the administered ligand with cellular ligands where iron chelates of sufficient liposolubility to penetrate cells are present. In order to look into some of these problems, several commercial hematinic chelates have been subjected to various conditions under which ionic iron might be liberated. A comparison has been made with inorganic Fe(II) and Fe(III), and chelates containing both Fe(II) and Fe(III) were included.

The possibility also exists of iron exchange between the administered chelate and the heme pigments, without liberation of ionic iron, since the porphyrins have such an extreme affinity for iron (as yet no measurement of iron-porphyrin stability constants has been possible). In this connection, however, Rubin (8) has shown that chelating agents of the EDTA type were unable to remove iron from siderophilin, and siderophilin was likewise unable to remove iron from the tighter chelates.

EXPERIMENTAL

Separation of Fe(II) and Fe(III) Ions.—The method of Stevens (9) was used with some modification. A chromatogram holder consisting of four glass tubes fitted with corks into which horizontal glass rods were inserted and held in place by rubber bands was constructed to avoid contamination from metal racks or clips. The developing solution was a mixture of 40 parts of *n*-butanol, 25 parts of ethanol, 25 parts of glacial acetic acid, and 35 parts of distilled water. The solvent was allowed to equilibrate in the tank overnight before use. Whatman No. 1 chromatography paper was cut into strips 15 in. by 2 in., washed with 2 *N* hydrochloric acid for 30 minutes, and rinsed with distilled water until a pH of 6.5 to 7 was reached. The strips were allowed to dry in air.

Solutions of $FeSO_4 \cdot 7H_2O$ and $Fe_2(NH_4)_2(SO_4)_2 \cdot 24H_2O$ were prepared to contain a concentration of approximately 1 mcg./μl. The pH of the ferrous sulfate solution was buffered to 4, and the ferric ammonium sulfate solution to 2.5, using saturated sodium acetate solution and sulfuric acid. This was done to minimize reduction and hydroxide formation. The solutions were spotted on the paper in 1 to 5 μl. amounts by means of a micropipet under a stream of warm air. The ferrous solution was spotted first and allowed to dry; the ferric solution was superimposed on the ferrous spot and allowed to dry. The strips were placed in contact with the developing solution and allowed to develop for 3.5 to 4 hours. The strips were removed, allowed to dry in air, and then exposed to water vapor containing a small amount of 8-hydroxyquinoline (oxine) from the side-arm of a distilling flask. The presence of Fe(II) and Fe(III) ions was indicated by green-black spots. The R_f value for Fe(III) was found to be 0.57, and that for Fe(II) was 0.24. The temperature was kept at 23–25°.

Preparation of a Standard Curve.—Exact quantities of 1 to 5 μl. of the standard solutions (containing 0.5 to 1.5 mcg. per μl.) were spotted on strips of Whatman No. 1 chromatography paper and placed in contact with the developing solution for 4 hours. The strips were removed and dried in air, and the spots were made visible by exposure to water vapor containing oxine. The strips were allowed to dry, and the maximum and minimum absorbances were determined using a Welsh Densichron densitometer, model 3853D, with a 3-mm. aperture. The minimum absorbance was that reading obtained from the paper itself. The average absorbance was determined by adding the maximum and minimum absorbances, dividing by two, and subtracting the paper blank. The areas of the spots were determined by tracing on graph paper and counting the squares, or by use of a planimeter.

Six to ten chromatograms were prepared for each known concentration, unsatisfactory results being discarded. An average of the results of the runs for each concentration was taken and the area multiplied by the average absorbance was plotted *vs.* the concentration.

Quantitative Determinations of Iron.—Each product or compound was analyzed under five different conditions: (*a*) untreated, (*b*) after treatment with stimulated gastric fluid U.S.P. XVI, (*c*) after treatment with simulated intestinal fluid U.S.P. XVI, (*d*) after treatment with 5 ml. of concentrated hydrochloric acid, and (*e*) after treatment with a solution of pH 7.5.

(*a*) *Untreated.*—An equivalent of the compound or product was diluted to 100 ml. with distilled water so that each microliter contained approximately 1 mcg. of iron. The paper strips were spotted in 1 to 5 μl. amounts and developed as described above. The average absorbances and areas of the spots were determined as described, and the concentration of iron read from the standard curve.

(*b*) *Treatment with Simulated Gastric Fluid.*—An equivalent of the compound or product was diluted to 100 ml. with simulated gastric fluid so that each microliter contained approximately 1 mcg. of iron. The solution was placed in a constant temperature bath for 1.5–2 hours at 37° with occasional agitation. The paper strips were spotted with 1 to 5 μl. amounts, and concentrations were determined.

(*c*) *Treatment with Simulated Intestinal Fluid.*—This procedure was carried out in the same manner as in (*b*).

(*d*) *Treatment with Concentrated Hydrochloric Acid.*—An amount of the compound or product was diluted with 5 ml. of hydrochloric acid and enough distilled water to make 100 ml. so that each microliter contained approximately 1 mcg. of iron. The solution was allowed to stand for 4 hours with occasional shaking, and paper strips were spotted with 1 to 5 µl. amounts. The remainder of the procedure was as described above.

(*e*) *Treatment with a Solution of pH 7.5.*—An amount of the product or compound was diluted to 100 ml. with an aqueous solution of pH 7.5, obtained with phosphate buffer, such that each microliter contained approximately 1 mcg. of iron. The solution was placed in a constant temperature bath at 37° for 1.5–2 hours with occasional agitation. The strips were spotted with 1 to 5 µl. amounts, and the concentrations of iron determined in the customary manner.

RESULTS AND DISCUSSION

In order to make comparisons of the amount of ionic iron liberated from commercial hematinic preparations under different conditions of acidity, a convenient assay procedure was required in which other components of the preparations, such as syrups, vitamins, coloring agents, calcium salts, phosphates, and the organic ligands, do not interfere. A chromatographic separation of Fe(II) and Fe(III) ions was carried out essentially as described by Stevens (9) except that ascending rather than descending development was employed. Quantitative estimation of the ionic iron liberated from the various commercial preparations and the inorganic iron salts selected as control compounds was based on techniques previously described by Block, Durrum, and Zweig (10). The method consisted primarily of measurement of area and absorbance of chromatographic spots of liberated ionic iron in the presence of oxine. A standard curve was drawn, plotting average absorbance times area *vs.* concentration and found to follow the Lambert-Beer law. A considerable number of volumes and concentrations were employed before optimum experimental conditions were determined.

Some difficulty was encountered in determining the areas of the spots, largely because of the contaminating ingredients present in the commercial preparations. Not only area, but also regularity and R_f value were affected, but employment of a sufficient number of determinations generally gave satisfactory average values. It was possible to determine ionic iron in amounts less than 0.5 mcg. per µl. by this procedure.

The relative amounts of ionic iron liberated under varying conditions of acidity: (*a*) without prior treatment, or after treatment with (*b*) simulated gastric fluid, (*c*) simulated intestinal fluid, (*d*) concentrated hydrochloric acid, and (*e*) aqueous solution of pH 7.5 were determined (see Table I). Treatment with concentrated hydrochloric acid was designed to liberate all of the iron present, and treatment with simulated gastric and intestinal fluids was carried out to determine effect of pH, pepsin, and pancreatin on the state of oxidation and release of ionic iron. Solutions of pH 7.5 were designed to show any effects due to pancreatin other than a pH effect. Treatment with simulated intestinal fluid and solution of pH 7.5 generally caused extreme tailing of the chromatographic spots, so these results are not included in Table I. The R_f value of the iron also was not reached in the former case, evidently due to combination of iron with pancreatin.

No significant change in oxidation state of iron was noted in any of the products under the conditions of acidity employed. Peptonized iron, ferrous gluconate, ferrous glycinate, and iron choline citrate all remained in the ferrous state, whereas the iron-carbohydrate complexes remained in the ferric state. The ferric complexes, as might be predicted, were the more stable and, in one instance, liberated no ionic iron even after treatment with simulated gastric fluid (pH 1.2). With the exception of the ferric-carbohydrate complexes, simulated gastric fluid liberated essentially all of the iron from the complexes studied.

It may be concluded that the more tightly bound ferric complexes are more successful in carrying iron through the gastrointestinal tract to the areas of absorption without loss of iron to pancreatin. Toxicity due to liberation of ionic iron should also be less with the more tightly bound complexes, and

Table I.—Assay Results of Various Iron Preparations

Product	Iron Present As	Oxidation State Found	Amount Recovered, %[a]		
			Hydrochloric Acid[b]	Untreated	Simulated Gastric Fluid
Livitamin with iron	Peptonized iron	+ +	97.00	59.86	108.47
Fergon compound elixir	Ferrous gluconate	+ +	90.00	76.99	90.85
Ferronord	Ferrous sulfate glycine complex	+ +	101.25	99.00	102.5
Imferon	Iron dextran complex	+ + +	92.40	0.0	25.80
Ferrolip	Iron choline citrate complex	+ +	96.00	69.50	94.67
Jefron elixir	Iron carbohydrate complex	+ + +	108.0	0.0	0.0
Saccharated iron oxide	Iron carbohydrate complex	+ + +	74.27	0.0	22.92
Ferrous sulfate[c]	...	+ +	99.63	100.0	97.64
Ferric ammonium sulfate	...	+ + +	99.51	100.0	98.06

[a] The percentages given are based upon the theoretical values given by the manufacturer. Iron concentrations could not be determined in alkaline media of pH 7.5 or simulated intestinal fluid. [b] The percentages given are the average values for a minimum of six determinations. [c] The percentages given are based upon the results of the findings of potassium permanganate assay.

there is already evidence to indicate that chelated iron is less toxic than ionic iron (4). In regard to subsequent distribution and utilization of iron, it may well be the case that chelation is a necessary requirement, since transport of ionic iron, *per se*, through cellular membranes is questionable. Release of iron from administered chelates to form hemoglobin should present no obstacle because of the known extreme affinity of porphyrins to chelate iron (11).

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Composition of Gum Turpentines of Pines

Pinus nelsonii and *Pinus occidentalis*

By NICOLAS T. MIROV, EUGENE ZAVARIN†, and JOSEPH G. BICHO†

It was found that the turpentine of *Pinus nelsonii* contained 49.60% *l*-α-pinene, 41.50% *l*-β-pinene, 1.02 dipentene, 0.24% camphene, and 0.01% *n*-heptane. The remainder contained 3.74% of oxygenated substances derived mainly from terpenes (including probably some oxygenated sesquiterpenes); about 0.02% of unidentified, low-boiling materials, pot residue, and losses. The possible occurrence in *P. nelsonii* turpentine of the oxygenated materials should be rechecked, using freshly collected material. The identification of camphene and *n*-heptane must be considered as tentative, only. The turpentine of *Pinus occidentalis* contained 63.8% *d*-, *dl*-α-pinene, 22.2% *l*-β-pinene, 7.7% *d*-Δ³-carene, 1.8% methyl chavicol, 1.1% dipentene, 0.5 % *d*-longifolene, 0.3% linalool, 0.2% camphene, and 0.1% *n*-heptane, with the remaining 2.3% accounted for by unidentified terpenes, pot residue, and losses. The identification of the last four constituents must be considered only tentative.

PINUS NELSONII Shaw is a Mexican piñon or nut pine, growing in a few restricted localities in the desert ranges of Mexican states Tamaulipas, Nuevo Leon, Coahuila, and San Luis Potosí. It is a small bushy tree with long pliant branches, sparse gray-green foliage, and gray bark. Its three cohering needles give the impression of a single, slender needle. A sample of the oleoresin of this pine was received from Aramberri, Nuevo Leon, Mexico. The trees from which the oleoresin was procured grew at an elevation of about 7000 ft. in Valle Hermoso, Miquihuana, Tamaulipas. Herbarium specimens accompanied the oleoresin sample.

Pinus occidentalis Swartz is a pine of the Caribbean area. It grows in eastern Cuba and on the Island of Hispañola, both in the Dominican Republic and in Haiti. It is closely related to *Pinus caribaea* and also to other pines of Mexico and of the southeastern United States. A generous sample of oleoresin of this pine was collected in August of 1960 at Mare Range section of the Pine Forest, Haiti, courtesy of SHADA.

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† Forest Products Laboratory, University of California, Richmond.

DISCUSSION AND EXPERIMENTAL

Pinus Nelsonii

The turpentine was distilled under reduced pressure. Toward the end of the operation, the pressure was reduced to 2 mm. and the temperature reached 215°. At the end, small quanitities of distillate solidified in the condenser. The pot residue was hard and brittle, showing that all volatile components, including sesquiterpenes and their derivatives, were removed from the oleoresin. Yield of turpentine was 27.8%; its physical characteristics were: density, d_4^{25}, 0.8557; index of refraction, n_D^{25}, 1.4711; and specific rotation, $[\alpha]_{578}^{23}$ −38.5. This was reported by Mirov (1).

A charge of 309.7 Gm. of *P. nelsonii* turpentine was distilled through a 90 cm. long, 12 mm. inside

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A charge of 309.7 Gm. of *P. nelsonii* turpentine was distilled through a 90 cm. long, 12 mm. inside

TABLE I.—FRACTIONAL DISTILLATION AND PHYSICAL PROPERTIES OF FRACTIONS OF *Pinus nelsonii* TURPENTINE, ATMOSPHERIC PRESSURE

Fraction No.	Temperature Range, °C.	% of Total	Density d_4^{25}	Refractive Index n_D^{25}	Specific Rotation $[\alpha]_D^{25}$ Detd. in 1958	Specific Rotation $[\alpha]_D^{25}$ Detd. in 1961	Main Components
1	145–155	1.98	0.8504	1.4622	−53.6	−43.24	α-Pinene
2	155–156	29.20	0.8515	1.4646	−52.1	−39.38	α-Pinene
3	156–157	10.10	0.8523	1.4660	−47.9	−42.11	α-, β-Pinene
4	157–158	7.36	0.8523	1.4672	−44.7	−39.33	α-, β-Pinene
5	158–159	3.55	0.8530	1.4687	−41.6	−39.99	α-, β-Pinene
6	159–160	3.88	0.8534	1.4700	−38.6	−33.20	β-, α-Pinene
7	160–161	6.14	0.8544	1.4713	−35.2	−22.03	β-, α-Pinene
8	161–162	4.58	0.8544	1.4727	−31.2	−19.35	β-, α-Pinene
9	162–163	9.82	0.8545	1.4740	−27.9	−23.11	β-Pinene
10	163–164	13.13	0.8605	1.4756	−25.2	−20.19	β-Pinene
Pot residue	>164	10.26	Redistilled under reduced pressure; see Table II				

TABLE II.—FRACTIONAL DISTILLATION AND PHYSICAL PROPERTIES OF FRACTIONS OF THE POT RESIDUE FROM *Pinus nelsonii* TURPENTINE DISTILLATION (TABLE I) AFTER STORAGE

Fraction No.	Temperature Range, °C.	Pressure, mm.	% of Total	Density d_4^{20}	Refractive Index n_D^{25}	Specific Rotation $[\alpha]_D^{25}$	Main components
11	61–66	22	1.03	0.8667	1.4782	−16.54	β-Pinene and dipentene
12	66–70	22	0.68	0.8671	1.4790	−13.61	β-Pinene and dipentene
13	70–74	22	0.71	0.8868	1.4814	− 4.43	β-Pinene and dipentene
14	74–115	22	0.77	0.9261	1.4891	+ 4.67	Oxygenated terpenes and dipentene
15	115–134	22	1.44	0.9180	1.4942	−15.33	Oxygenated terpenes
16	134(22)–64(2)	22–2	0.90	0.9104	1.4968	− 9.71	Oxygenated terpenes
17	64–95	2	0.31	0.9046	1.4972	...	Oxygenated terpenes
18	95–118	2	0.29	0.9571	1.5008	−16.09	Oxygenated terpenes and sesquiterpenes
19	up to 172	1.5	0.26	0.9527	1.5038	...	Oxygenated sesquiterpenes
Pot residue			3.07				Final bath temperature 225° at 1.5 mm. pressure
Loss			0.80				
Total			10.26				

diameter Todd column packed with $^3/_{32}$-in. diam. glass helices. A 5:1 reflux ratio was maintained throughout the operation. The results of distillation and the physical constants of the fractions are shown in Table I.

An unusual feature of the physical properties was very high levo-rotation of the α-pinene (fractions 1 and 2). Never before have we encountered such a high rotation of α-pinene. After distillation, the 10 fractions and the residue were kept in air-tight containers for $2^1/_2$ years at 5°. No doubt some oxidation and other changes in the material occurred during the storage period, as indicated by the rotation figures in columns 6 and 7. Even so, the specific rotation of fraction 1 has remained rather high.

After $2^1/_2$ years storage, the pot residue of the fractional distillation amounting to 31.8 gm. was distilled under reduced pressure, using a 90 cm. long, 4 mm. inside diameter wire spiral column. A 10:1 reflux ratio was maintained during fractionation. The results are shown in Table II.

Preliminary identification of components and determination of the quantitative composition of all 19 fractions was made by vapor-phase chromatography after the storage period. The Aerograph model A-90-C used was equipped with automatic integrator and with 5 ft. of Silicone 550–20% on chromosorb and 5 ft. Ucon polar 20% on firebrick columns, as recommended by Stanley.[1] Helium was used as the mobile gas phase. The results are shown in Tables III, IV, and VIII. In all cases, samples of known materials were used for comparison of relative retention volumes, which were computed on the basis of the time necessary for α-pinene to appear as equal to 1.0. In quantitative work, the peak areas were assumed to correspond directly to the weight percentages of the individual compounds comprising the fraction under consideration. It was felt that sufficient accuracy was attained as, in most cases, compounds comprising individual fractions did not differ too much structurally. The results are shown in Tables III and IV. In addition to the identified components, unidentified peaks were noted in fractions 1 and 13–19.

The identifications of α-pinene, β-pinene, and dipentene were substantiated by preparation of

[1] Dr. R. G. Stanley, U. S. Forest Service, personal communication.

crystalline derivatives, as given in Table V. *n*-Heptane and camphene were identified only on the basis of relative retention volumes and boiling points, and for this reason their presence must be regarded as established only tentatively.

Of the rather large number of unknown peaks encountered, five were singled out because of either their intensity or their characteristic position in vapor-phase chromatographic experiments.

Peaks A and B from fraction 1 (relative retention volumes of 0.30 and 0.34; Ucon polar column; T = 130°; 29 ml./min.) appear near *n*-heptane and could easily correspond to its homologs.

Peak C from fractions 14 and 15 appeared as a singlet at 210° (17.6 ml./min., Silicone 550), with relative retention volume of 2.13 and as a doublet at 152° (22.0 ml./min., Silicone 550), with relative retention volumes of 3.1 and 3.5. Its infrared spectrum obtained on a smear from the vapor-phase-chromatography condensate indicated, among other groups, the presence of a hydroxyl group (band at 3450 cm.$^{-1}$), a $CH_2 = CR_2$ group (bands at 3100, 1417, and 890 cm.$^{-1}$), a carbonyl group (band at 1715 cm.$^{-1}$, the position agreeing best with a non-conjugated keto group either in straight chain or in six-membered ring), and probably an isopropyl group (doublet at 1390 and at 1374 cm.$^{-1}$). Apparently the material represented a mixture of an alcohol and a ketone. *p*-Nitrobenzoylation of fraction 4 yielded a small amount of *p*-nitrobenzoate, which melted after recrystallization from ethanol at 53–54°. 2,4-Dinitrophenylhydrazone was formed from the same fraction, but in an amount too small for any further work. Examination of all the data obtained indicated that the alcohol in fraction 4 was not identical with any terpene alcohol recorded in pine turpentines.

Peak D, present in fractions 15–18, appeared at a relative retention volume of 4.03 (Silicone 550, 210°, 17.6 ml./min.). The infrared spectrum indicated the presence of a hydroxyl group (band at 3420 cm.$^{-1}$), a double bond (bands at 3050 and 1650–1690 cm.$^{-1}$), and probably an isopropyl group (doublet at 1375 and at 1390 cm.$^{-1}$).

Peak E, present in the same fractions, appeared at the relative retention volume of 5.09 when run under the same conditions. The infrared spectrum indicated the presence of a hydroxyl group (band at

TABLE III.—VAPOR-PHASE CHROMATOGRAPHIC ANALYSIS OF THE TURPENTINE OF *Pinus nelsonii* EXCLUDING POT RESIDUE FROM TABLE I

Fraction No.	% of Total	α-Pinene, %	Camphene, %	β-Pinene, %
1[a]	1.98	85.0	0.6	12.5
2	29.20	91.8	0.2	8.0
3	10.10	78.8	0.2	21.0
4	7.36	68.8	0.2	31.0
5	3.55	56.0	1.0	43.0
6	3.88	44.7	0.7	54.6
7	6.14	35.5	0.5	64.0
8	4.58	23.2	0.4	76.4
9	9.82	8.7	tr.	91.3
10[b]	13.13	1.3	tr.	97.5
Pot residue	10.26	see Table IV		

[a] *n*-Heptane, 0.6%; peak A, 0.1%; peak B, 1.2%. [b] Dipentene, 1.2%.

TABLE IV.—VAPOR-PHASE CHROMATOGRAPHIC ANALYSIS OF THE TURPENTINE OF *Pinus nelsonii*, POT RESIDUE FROM TABLE I, AFTER STORAGE[a]

Fraction No.	% of Total	α-Pinene, %	β-Pinene, %	Dipentene, %	Peak C, %	Peak D, %	Peak E, %	Sum of Other Peaks, %
11	1.03	1.0	84.3	14.7	...	...	...	...
12	0.68	0.2	75.6	24.2	...	...	...	...
13	0.71	...	47.8	45.7	...	...	...	6.5
14	0.77	...	7.3	24.1	54.1	...	...	14.5
15	1.44	...	...	2.8	14.8	31.1	45.7	5.6
16	0.90	...	...	...	...	19.9	56.0	24.1
17	0.31	...	...	...	...	13.5	55.0	31.5
18	0.29	...	...	...	...	2.5	30.6	66.9
19	0.26	...	...	...	...	...	...	100.0

[a] The composition of *P. nelsonii* turpentine, calculated from Tables III and IV, is as follows: 0.01% *n*-heptane; 49.60% α-pinene; 41.50% β-pinene; 0.24% camphene; 1.02% dipentene; traces peak A; 0.02% peak B; 0.63% peak C; 0.68% peak D; 1.41% peak E; 1.02% sum of other high-boiling materials; 3.07% pot residue; 0.80% loss.

TABLE V.—IDENTIFICATION OF THE COMPONENTS OF TURPENTINE FROM *Pinus nelsonii*

Terpene	Derivative	Prepared from Fraction No.[c]	M.p., °C.	Mixed M.p.	Other Identification[a]
n-Heptane[b]	...	..	...	...	...Bp, VP
α-Pinene	Pinonic acid semicarbazone	2	204–205	...	...Bp, n, d, α, VP
Camphene[b]	...	..	...	...	...Bp, VP
β-Pinene	Nopinic acid	10	127.8–128.8	no depression	Bp, n, d, α, VP
Dipentene	Tetrabromide	13	121–122	no depression	Bp, n, d, α, VP

[a] Bp = boiling point, n = refractive index, d = density, α = specific rotation $[\alpha]^{25}_{D}$, VP = vapor-phase chromatography. [b] *n*-Heptane and camphene were identified only on the basis of relative retention volumes and boiling points; thus their presence must be regarded as established only tentatively. [c] See Tables I, II and IX.

3400 cm.$^{-1}$), a $CH_2{=}CR_2$ nonconjugated double bond (bands at 3090, 1635, and 885 cm.$^{-1}$), and possibly an isopropyl group (doublet at 1387 and at 1373 cm.$^{-1}$).

Preparation of hydrochloride from either fraction 15 or fraction 16 resulted only in noncrystallizable oils. Fraction 19 gave a mixture of hydrochlorides melting indenfinitely between 99 and 110°, even after three recrystallizations. Fraction 16 gave a fairly good yield of *p*-nitrobenzoate which, after recrystallization from alcohol and following purification with charcoal, sublimed between 190 and 232°, without melting. As the ester would be expected to have a vapor pressure to high for sublimation, it is possible that *p*-nitrobenzoic acid was eliminated and sublimed during the melting. The ester obtained probably was derived from the compound of Peak E, as the latter is the main constituent of fraction 16.

The infrared spectra of the remaining fractions 17, 18, and 19, all showed the presence of hydroxyl compounds.

As considerable time elapsed after fractionation and the determination of physical constants on the fractions before the vapor-phase chromatographic analysis and characterization of the constituents were performed, the specific rotations of the 10 fractions were redetermined. In all cases, a considerable drop in rotation values was noted. Shaking fractions 2 and 10 with potassium iodide solution liberated iodine. This suggested that air oxidation had taken place during the storage period and cast doubt as to whether the oxygenated terpenes of fractions 14–19 were present in the original turpentine. As no fresh *Pinus nelsonii* turpentine was available to check this point, the work on these materials was abandoned.

The composition of *Pinus nelsonii* turpentine differs considerably from that of the other five piñon pines in that this species contains a large percentage of *l*-β-pinene (1). Its α-pinene possesses unusually high rotatary power. It would be interesting to compare the turpentine of this piñon with the turpentine of the new piñon pine discovered in high mountains of Nuevo Leon, Mexico (2).

Pinus occidentalis

The turpentine was obtained by heating 3.5 Kg. of the oleoresin under reduced pressure, so that at the end of the operation the temperature of the charge reached 190° while the pressure was decreased to 3.4 mm. of mercury.

This operation was performed by the personnel of the Pacific Southwest Forest Experiment Station at Placerville, Calif.

Yield of turpentine was 20.0%; the flask residue (*i.e.*, rosin) was hard and brittle.

The turpentine possessed the following characteristics: density, d_4^{25}, 0.859; index of refraction, n_D^{25}, 1.4688; and specific rotation, $[\alpha]_D^{25}$ + 10.88

A charge of 425 Gm. of the turpentine was fractionated in a 90 cm. long, 25 mm. inside diameter Stedman column. A 10:1 reflux ratio was maintained during fractionation. The results are shown in Table VI.

The preliminary identification of components and determination of the quantitative composition of

TABLE VI.—FRACTIONAL DISTILLATION AND PHYSICAL PROPERTIES OF THE FRACTIONS OF *Pinus occidentalis* TURPENTINE

Fraction No.	Temperature Range, °C.	Pressure, mm.	% of Total	Density, d_4^{25}	Refr. Index n_D^{25}	Specific Rotation $[\alpha]_D^{25}$	Main components
1	100.0–102.0	154.0	1.15	0.856	1.4597	+20.53	α-Pinene
2	102.0–102.0	154.0	26.25	0.857	1.4637	+21.26	α-Pinene
3	72.5–72.5	51.5	11.30	0.857	1.4637	+21.20	α-Pinene
4	72.5–72.7	51.5	12.70	0.853	1.4640	+20.89	α-Pinene
5	72.7–74.5	51.5	6.60	0.855	1.4653	+16.82	α-Pinene
6	74.5–77.0	51.5	5.20	0.860	1.4691	+ 4.57	α-Pinene and β-pinene
7	77.0–78.8	51.5	7.55	0.861	1.4729	− 8.46	β-Pinene and α-pinene
8	78.8–79.8	51.5	10.25	0.861	1.4757	−17.50	β-Pinene
9	79.8–81.3	51.5	2.70	0.862	1.4756	−14.18	β-Pinene and Δ^3-carene
10	81.3–83.0	51.5	1.30	0.862	1.4744	− 7.11	β-Pinene and Δ^3-carene
11	83.0–84.5	51.5	5.50	0.860	1.4716	+ 8.97	Δ^3-Carene and β-pinene
12	66.5–66.5	20.5	2.35	0.856	1.4716	+11.54	Δ^3-Carene and dipentene
13	66.5–68.0	20.5	1.12	0.851	1.4764	− 1.70	Dipentene, Δ^3-carene, and linalool
14	up to 125	4.0	2.57	0.951	1.5009	+ 6.69	Methyl chavicol and longifolene
Trap	(dry ice-acetone)	...	1.30	0.858	1.4686	+ 8.79	α-Pinene, β-pinene, and Δ^3-carene
Pot residue	...	...	1.25	...	...	...	Heated up to 215° at 4 mm.
Loss	...	...	0.77	...	...	...	

TABLE VII.—VAPOR-PHASE CHROMATOGRAPHIC ANALYSIS OF THE TURPENTINE OF *Pinus occidentalis*[a]

Fraction No.	% of Total	*n*-Heptane, %	α-Pinene, %	Camphene, %	β-Pinene, %	Δ³-Carene, %	Dipentene, %	Linalool, %	Methyl Chavicol, %	Longifolene, %	Unidentified, %
1	1.5	...	100.0	...	...	...	...	...	...	...	...
2	26.25	...	100.0	...	...	...	...	...	...	...	...
3	11.30	...	100.0	...	...	...	...	...	...	...	...
4	12.70	...	98.0	0.5	1.5	...	...	...	...	...	...
5	6.60	...	88.5	1.5	10.5	...	...	...	...	...	...
6	5.20	...	59.0	0.5	40.5	...	...	...	...	...	...
7	7.55	...	29.75	0.25	70.0	...	...	...	...	...	...
8	10.25	...	7.0	...	93.0	...	...	...	...	...	...
9	2.70	...	...	...	81.0	19.0	...	...	...	..	...
10	1.30	...	...	...	67.0	33.0	...	...	...	...	...
11	5.50	...	...	...	21.0	79.0	...	...	...	...	...
12	2.35	...	...	...	...	84.0	16.0	...	...	...	...
13	1.12	...	...	...	...	18.0	61.0	15.0	...	...	6.0
14	2.57	...	...	...	...	0.5	1.0	3.0	71.5	17.5	6.5
Trap	1.30	6.5	56.0	...	20.0	16.5	1.0	...	...	...	...

[a] The composition of *P. occidentalis* turpentine, calculated from the above analysis, is as follows: 0.1% *n*-heptane; 63.8% α-pinene; 0.2% camphene; 22.2% β-pinene; 7.7% Δ³-carene; 1.1% dipentene; 0.3% linalool; 1.8% methyl chavicol; 0.5% longifolene; 0.2% unidentified; 1.3% pot residue; 0.8% loss.

TABLE VIII.—RELATIVE RETENTION VOLUMES OBTAINED BY VAPOR-PHASE CHROMATOGRAPHY OF COMPONENTS OF TURPENTINES FROM *Pinus nelsonii* AND *P. occidentalis*

Terpene	Silicone 550 20% on Chromosorb; 5 ft. Col., 20 ml./min. R.R.V.[a]	Temp., °C.	Ucon Polar 20% on Firebrick; 5 ft. Col., 30 ml./min. R.R.V.[a]	Temp., °C.
n-Heptane	0.31	130	0.24120	
α-Pinene	1.00	130	1.00120	
Camphene	1.15	130	1.21	120
β-Pinene	1.35	130	1.44	120
Δ³-Carene	1.48	130	1.70	120
Dipentene	1.67	130	2.09	120
Linalool	2.34	130	2.98	120
Methyl chavicol	2.94	200	4.75	200
Longifolene	5.88	200	7.66	200

[a] Relative retention values.

the fractions obtained was determined by vapor-phase chromatography, as described above. The results are shown in Tables VII and VIII. In addition to identified components, several small unidentified peaks were noted in fractions 13 and 14.

The specific rotations of individual terpenes were computed from the rotation data in Table VI and from vapor-phase chromatography results, considering also the melting points of the prepared derivatives. The compounds were then designated as *d* or *l*, if the optically active material represented at least 80% of the total, and as *d*, *dl* or *l*, *dl* if it was less than 80% and more than 0% of the total. The maximum rotation values for individual terpenes, given in the literature, were used in this computation.

The identification of *d*, *dl*-α-pinene, *l*-β-pinene, *d*-Δ³-carene, dipentene, and methyl chavicol was substantiated by preparation of crystalline derivatives, as given in Table IX. Because of their small amounts, *n*-heptane, camphene, and linalool were identified only on the basis of relative retention volumes and, where possible, of constants from Table VI. For this reason, their identification must be regarded as only tentative. In the case of longifolene, the material was identified by physical constants and relative retention volumes and also by infrared spectrum on the sample collected by condensation of the corresponding fraction separated by vapor-phase chromatography.

The close relationship of *Pinus occidentalis* to *P. caribaea* is manifested by the presence of Δ³-carene in both pines (1).

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TABLE IX.—IDENTIFICATION OF THE COMPONENTS OF *Pinus occidentalis* TURPENTINE

Terpene	Derivative	Fraction	M.p., °C.	Other Criteria[a]
n-Heptane	...	Trap	...	Bp, VP
α-Pinene	Nitrosochloride	2	102.8 (decompn.)[b]	Bp, n, d, α, VP
Camphene	...	4, 5, 6, 7	...	Bp, VP
β-Pinene	Nopinic acid	8	124.0–125.8[b]	Bp, n, d, α, VP
Δ³-Carene	Nitrosate	11	146.2 (decompn.)[b]	Bp, n, d, α, VP
Dipentene	Tetrabromide	13	121.8–122.6[b]	Bp, n, d, α, VP
Linalool	...	13	...	Vp, Bp
Methyl chavicol	Anisic acid	14	181.8–183.0[b]	Bp, n, d, α, VP
Longifolene	...	14	...	Vp, Bp, α, IR

[a] Bp = boiling point, n = refractive index, d = density, α = specific rotation $[\alpha]_D^{25}$; VP = vapor-phase chromatography, IR = infrared spectroscopy. [b] Mixed melting point, no depression.

Structural Studies on the Triterpenes of *Schinus terebinthefolius*

By KRISHAN K. KAISTHA† and LEMONT B. KIER

A second triterpene, schinol (II), has been isolated from the berries in the ether extract. The relationship of II to previously reported terebinthone (I), has been demonstrated. Terebinthone (I) has been proven to be a triketo triterpene and schinol (II) to be a dihydroxy, keto triterpene. A unique type of dehydration has been discovered in II and a possible mechanism is discussed. Structures for I and II are proposed.

THE partial structural elucidation of a triterpene, terebinthone (I), isolated from the berries of *Schinus terebinthefolius* (Radd.), has been previously described (1). Terebinthone was found to be a 3,11-diketotriterpene with an ethylidene group in the side chain. The nature of a third oxygen function and an element of unsaturation were in doubt but a cyclic ether was proposed. In this present work, the authors have isolated a second triterpene, schinol (II), from this plant and present proposed structures for both compounds.

The triterpene (II), provisionally named schinol from the genus name of the plant, was found to be a $C_{30}H_{50}O_3$ dihydroxy ketone with one reactive double bond. A derivative of the two hydroxyl groups was prepared, but a derivative of the carbonyl group could not be prepared. This indicated that the carbonyl group was in a hindered position. This belief was substantiated by the presence of the carbonyl absorption band at 1690 cm.$^{-1}$ in the infrared which is characteristic for hindered carbonyl groups (2, 3). Except for the hydroxyl and carbonyl group regions, the infrared spectra of schinol (II) and terebinthol (I) were practically identical.

The presence of three carbonyl groups in terebinthone (I) was concluded by comparing the molar absorptivities, ϵ, of the compounds in Table I at 285 mμ. This wavelength was selected as being characteristic of isolated carbonyl groups in cyclohexanones (4). The value of ϵ for schinol was in close agreement with the values reported for one carbonyl group. The two compounds, 3-hydroxy-terebinthone (III) and 3-desoxydihydroterebinthone (IV) had ϵ values corresponding to two carbonyl groups. The ϵ value for terebinthone was in agreement with the presence of three carbonyl groups. These findings indicated that I and II might well be interconvertible or that both might be converted to a common intermediate.

TABLE I.

Compound	Gm./L., Dioxane	ϵ	Estd. No. of C=O
Schinol	4.06	14	1
3-Hydroxy-terebinthone	3.95	28	2
3-Desoxydihydro-terebinthone	4.05	28	2
Terebinthone	4.03	48	3

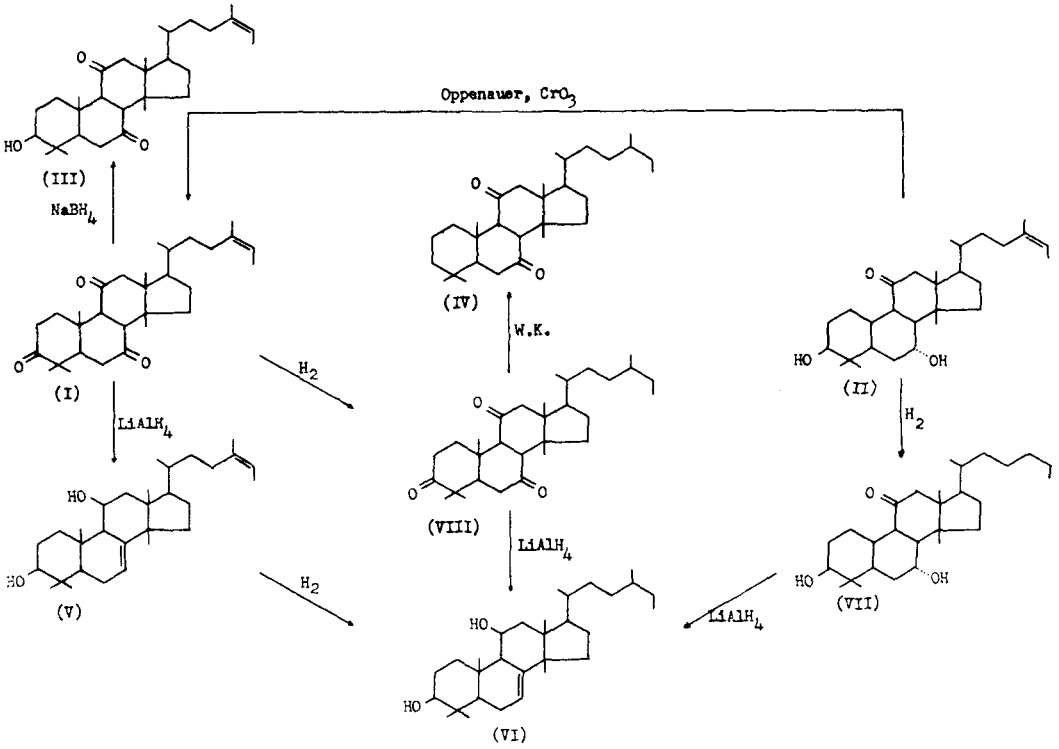
Oxidation with a chromium trioxide-pyridine complex (5) of II to I was successful though the yield was small. A higher yield was obtained by employing the Oppenauer method (6) with aluminum tertiary-butoxide. This gave conclusive evidence that terebinthone was a triketo compound.

It was necessary to re-examine terebinthol (V) and dihydroterebinthol (IV) since these were previously suspected to be dihydroxy ethers (1). If terebinthone is a triketone then hydride reduction should give a triol. Accordingly V and VI were carefully purified by repeated chromatography until a sharp consistent melting point was obtained. Elemental analyses now gave values which did not agree with those previously reported or those of the expected triol and dihydrol triol. Instead the values obtained for V and VI, $C_{30}H_{50}O_2$ and $C_{30}H_{52}O_2$ indicated that both compounds were missing the elements of water from the expected values. Peracid titration (7) indicated that a new double bond had been introduced in each compound.

In an effort to determine if this unusual mechanism was operative in schinol (II), dihydroschinol (VII), from catalytic hydrogenation of schinol, was subjected to the same lithium aluminum hydride reduction previously reported (1). This gave a diol missing the elements of water from the expected formula. A comparison showed that this compound was identical with VI.

The conversion of I and II to a common product VI conclusively proved that terebinthone (I) and schinol (II) possessed the same ring system, side chain, and location of oxygen

Received February 19, 1962, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Florida, Gainesville.
Accepted for publication March 20, 1962.
† Abstracted, in part, from a dissertation for the Ph.D. degree. Winner of a Lunsford-Richardson award.



functions. It was also obvious that the location of the newly introduced double bond in both V and VI would be invaluable in locating the position of the remaining oxygen function, if it was assumed the 3,11 assignment was correct.

Attempts to hydrogenate the new double bond in V and VI were unsuccessful which narrowed the possibilities to the Δ^7 and Δ^8 positions of a tetracyclic triterpene (8, 9, 10). Both V and VI gave negative Tortelli-Jaffe tests specific for a Δ^8 or a Δ^7 which can migrate to a $\Delta^{8,14}$ (11). This latter possibility can be discarded since this migration in a tetracyclic triterpene is prevented by the presence of a 14-methyl group. The nature of this double bond was further studied by plotting its molar absorptivity, ϵ, over the 205–225 mμ region (7). See Fig. 1. Samples of Δ^8-lanostanol and Δ^7-ergostene were used for direct comparison. Both Δ^7-ergostene and VI fell in the same region assigned to trisubstituted double bonds, while Δ^8-lanostanol fell in the same region assigned to tetrasubstituted double bonds (12, 13). Thus, it was concluded that the new double bond introduced in V and VI from hydride reduction is in the Δ^7 position.

In terebinthone (I) the three carbonyl groups must be in the ring system since the only unhindered group is in the 3 position. The infrared spectrum of terebinthone gave no evidence of a carbonyl group in ring D (14) hence the three six-membered rings were the only possible sites. Furthermore, the infrared and ultraviolet spectra of terebinthone (I) showed no absorption due to adjacent carbonyl groups (15) which indicated that the three carbonyl groups must be distributed in rings A, B, and C of the tetracyclic triterpene structure. This is consistent with the assignment of the new double bonds in V and VI to the Δ^7 position since this double bond must have been formed by a reduction of the carbonyl group at C-7 followed by a dehydration.

The possibilities that schinol (II) might be a 3,11-α-dihydroxy-7-keto or 3,11-β-dihydroxy-7-keto triterpene were considered but rejected. If the 11-β-hydroxyl group was present it would be unacylable (1). Schinol formed a derivative of both hydroxyl groups. If the 11-α-hydroxyl group was present a derivative of both hydroxyl groups could be prepared but hydride reduction of dihydroterebinthone (VIII) would not give dihydroschinol (VII) since lithium aluminum hydride reduction of an 11-keto group gives almost exclusively the 11-β-hydroxy compound (16, 17, 18).

These facts present the interesting conclusion that lithium aluminum hydride dehydrates a 7-hydroxyl group in schinol (II) while in terebinthone (I), this reagent first must reduce the carbonyl group to a 7-hydroxyl group then de-

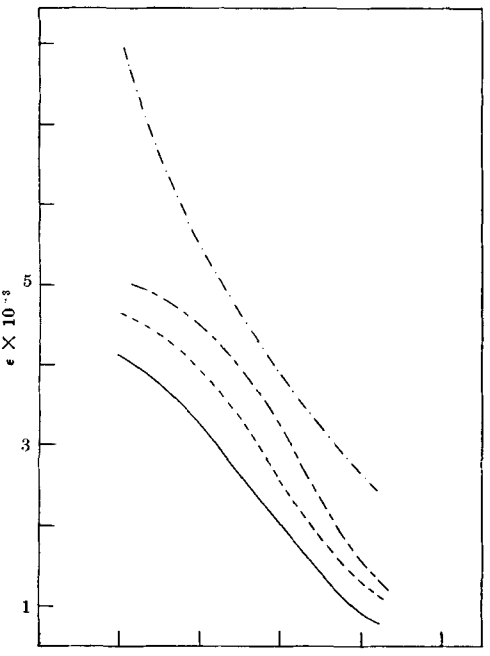


Fig. 1.—Ultraviolet absorption of —— dehydrodihydroschinol (VI), — · — lanostenol, — - — 7-ergostenol, - - - dihydroterebinthol (VI).

hydrate it by a similar mechanism. There is no precedence for this reaction in the literature though recent work (19), has shown that 3-α-*p*-toluene sulfonates of steroids can be dehydrated with lithium aluminum hydride to Δ^2 compounds very readily. This is a *trans* elimination with the large ester forming the leaving group and the hydride abstracting the adjacent proton. On this basis we would like to propose a similar mechanism (IX) with some form of an aluminum alcoholate forming the leaving group and a second mole of the hydride serving as the proton abstracting base. If this likely mechanism is operative, then the 7-hydroxyl group involved must be α-oriented, for a 7-β-hydroxyl group would be cisoid to the 8-hydrogen and a *cis* elimination mechanism would probably proceed with much more difficulty, if at all, at 36°.

There remained the necessity of proving the orientation of the 3-hydroxyl group of schinol (II). This was determined by treating dihydroschinol (VII) with phosphorus pentachloride. If the 3-β-hydroxyl group is present then X should suffer ring contraction with the formation of an isopropylidene group (XI) (20). Ozonolysis of the product from treatment of VII gave acetone proving a 3-β-hydroxyl group. This assignment of a β orientation is consistent with the fact that hydride reduction of dihydroterebinthone (VIII) should give almost exclusively a 3-β-hydroxyl group (21) which resulted in a compound VI which could also be obtained from schinol (II).

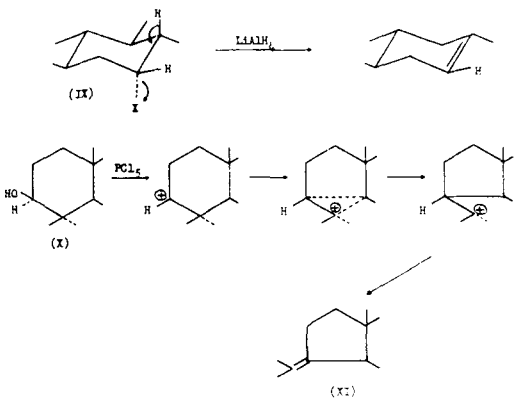
From these findings the authors propose the structures I and II to terebinthone and schinol, respectively.

EXPERIMENTAL[1]

Isolation of Schinol (II).—The hexane marc of the berries of *Schinus terebinthefolius* was extracted with ether in a Soxhlet extractor for 24 hr. The ether was allowed to evaporate leaving a crude crystalline residue. This residue was crystallized several times from ethyl acetate to give a 0.35% yield of schinol, m.p. 145–146.5°, $[\alpha]_D^{23}$ -39.7° (chloroform).

Anal.—Calcd. for $C_{30}H_{50}O_3$: C, 78.55; H, 10.99; 4 C-CH_3, 13.10. Found: C, 78.74; H, 10.74; C-CH_3, 11.98 (Kuhn-Roth).

Schinol was found to be slightly soluble in hexane, ethanol benzene, and chloroform but insoluble in aqueous acid and base. Infrared analysis in nujol showed absorption at 3500^{-1} cm. (hydroxyl), 1700^{-1} cm. (carbonyl), and 1645^{-1} cm. (alkene). The region 1400–650^{-1} cm. was practically identical to that of terebinthone (I). Schinol (II) gave positive Liebermann-Burchard, Salkowski, and tetranitromethane tests but gave negative Zimmermann and Tortelli-Jaffe tests.



Schinol Dibenzoate.—One gram of schinol in 10 ml. of anhydrous pyridine was treated with 2 ml. of benzoyl chloride and the product isolated in the usual way, m.p. 125–127°.

Anal.—Calcd. for $C_{44}H_{58}O_5$: C, 79.24; H, 8.77. Found: C. 79.63; H, 9.11.

Perebenzoic Acid Titration of Schinol (II).—The titration was conducted according to the method previously described (7). The perbenzoic acid consumed was equivalent to 1.2 double bonds.

Dihydroschinol (VII).—A solution of 0.8 Gm. of schinol (II) in 50 ml. of ethyl acetate was hydrogenated at atmospheric pressure using pre-reduced platinum oxide as a catalyst. The consumption of hydrogen was equivalent to 94% of the theoretical amount for one double bond. The ethyl acetate was removed under a vacuum and the residue crystallized from benzene to give 0.78 Gm. of VII, m.p. 148.5–150°, $[\alpha]_D^{23}$-37.3° (chloroform).

[1] All melting points were taken on the Kofler apparatus. Analyses were performed by Galbraith Analytical Laboratories, Knoxville, Tennessee and Weiler and Strauss Microanalytical Laboratories, Oxford, England.

Anal.—Calcd. for $C_{30}H_{52}O_3$: C, 78.20; H, 11.38; two active hydrogens, 0.43. Found: C, 78.53; H, 11.12; active hydrogen, 0.47.

The compound gave no tetranitromethane test and showed no infrared absorption for a double bond.

3-Hydroxy-terebinthone (III).—The method described by Rao (22) was used to reduce 1.5 Gm. of terebinthone (I). The reduction was conducted with sodium borohydride in aqueous dioxane. The crude product was chromatographed on a Celite-magnesium trisilicate (1:1) column, eluting with hexane. The purified compound was crystallized from benzene, m.p. 197–199°, $[\alpha]_D^{23}$ -23.4° (chloroform).

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.89; H, 10.59. Found: C, 78.84; H, 10.73.

The infrared spectrum showed absorption at 3400 cm.$^{-1}$ (hydroxyl) and 1690 cm.$^{-1}$ (carbonyl). The compound gave no Zimmermann reaction.

3-Hydroxy-terebinthone Mono-*p*-nitrobenzoate.—A quantity of III was converted to the *p*-nitrobenzoate in the usual way. The crude product was chromatographed on alumina and crystallized from methanol to give crystals, m.p. 115–117° (capillary).

Anal.—Calcd. for $C_{37}H_{51}NO_6$: N, 2.31. Found: N, 2.55.

The infrared analysis showed no absorption for an hydroxyl group.

Ultraviolet Studies of I, II, III, and IV.—The ultraviolet absorptions of terebinthone (I), schinol (II), 3-hydroxy-terebinthone (III), and 3-desoxy-terebinthone (IV) were measured at 285 mμ using 0.01 molar concentrations in dioxane. The molar absorptivities, ϵ, were calculated. See Table I.

Chromic Acid Oxidation of II to I.—A solution of 1 Gm. of schinol (II) in 20 ml. of anhydrous pyridine was added to a solution of 1 Gm. of chromium trioxide in 20 ml. of anhydrous pyridine. The solution was allowed to stand for 24 hours at room temperature. It was then diluted with 100 ml. of water and extracted with ethyl acetate and this in turn washed with dilute acid. The ethyl acetate was evaporated and the residue chromatographed on a Celite-magnesium trisilicate (1:1) column. A crystalline product weighing 50 mg. was obtained, m.p. 177–179°. A mixed melting point with terebinthone (I) gave no depression and the infrared spectra were identical.

Oppenauer Oxidation of II to I.—A solution of 4 Gm. of schinol (II) and 40 ml. of freshly distilled cyclohexanone in 400 ml. anhydrous toluene was distilled until 150 ml. of toluene was removed. To the remaining solution was added dropwise, 4 Gm. of aluminum tertiary-butoxide in 150 ml. of dry toluene. This solution was refluxed 45 minutes then allowed to stand 12 hours. The reaction mixture was then diluted with water and steam distilled to remove volatile solvents. The precipitate which formed was filtered off then chromatographed on Celite-magnesium trisilicate (1:1). The hexane eluate was recrystallized from ethanol to give 2.5 Gm. of crystals, m.p. 178–179°. A mixed melting point with terebinthone (I) gave no depression and the infrared spectra were identical.

Purification of Terebinthol (V).—A 0.5-Gm. quantity of terebinthol (V), previously prepared, (1) was chromatographed several times on columns of Celite-magnesium trisilicate (1:1) until a fraction was obtained, m.p. 192–193°.

Anal.—Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38. Found: C, 81.66; H, 11.44.

Purification of Dihydroterebinthol (VI).—This compound, previously prepared (1), was purified as described for terebinthol (V) until a fraction m.p. 160-161° was obtained.

Anal.—Calcd. for $C_{30}H_{52}O_2$: C, 81.02; H, 11.79. Found: C, 81.14; H, 11.69.

Reduction of Dihydroschinol (VII) to VI.—Dihydroschinol (VII) was reduced with lithium aluminum hydride in ether by the same procedure previously described (1). Purification on a Celite-magnesium trisilicate (1:1) column gave a crystalline product which did not depress the mixed melting point with dihydroterebinthol (VI) and the infrared spectra were identical.

Ultraviolet Analysis of Dihydroterebinthol (VI).—The ultraviolet spectrum of dihydroterebinthol (VI) was obtained in the region 205–225 mμ and compared with the spectra of Δ^8-lanostanol and Δ^7-ergostene. See Fig. 1.

Retropinacol Rearrangement of Dihydroschinol (VII).—A solution of 1.5 Gm. of dihydroschinol (VII) in 150 ml. of methylene chloride was treated with 0.75 Gm. of phosphorus pentachloride. This solution was stirred for 2 hours then refluxed for 1 hour. The reaction mixture was then added to 50 ml. of water and steam distilled to remove the solvent and hydrolyse the phosphorus pentachloride. The residue remaining weighed about 1 Gm. and could not be purified.

Ozonolysis of Crude Retropinacol Products (XI).—One gram of the crude retropinacol product (XI) in 30 ml. of carbon tetrachloride at 0° was treated with a stream of 3% ozonized oxygen for 30 minutes. Water and a few pieces of zinc were then added and the mixture steam distilled into an aqueous acid solution of 2,4-dinitrophenylhydrazine. From this solution was isolated the corresponding acetone derivative, confirmed by a mixed melting point with the authentic compound.

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Veratrum Alkaloids L

Structure-Activity Relationships in a Series of Synthetic Hypotensive Analogs of the Protoveratrines

By S. MORRIS KUPCHAN, EIICHI FUJITA, JOHN C. GRIVAS, and LAWRENCE C. WEAVER

A study aimed at further elucidation of the relationship between hypotensive activity and structure among analogs of the protoveratrines is reported. A series of synthetic protoverine tetraesters, which differ from each other only in the nature of the acid residue affixed at C_{15}, have been prepared and subjected to preliminary pharmacological evaluation. The results indicate that alteration in the structure of the ester affixed at C_{15} in analogs of the protoveratrines profoundly affects hypotensive potency.

Two recent developments have stimulated studies involving the synthesis and pharmacological evaluation of analogs of the protoveratrines. On the chemical side, our elucidation of the complete structures and configurations of protoveratrine A (I) and protoveratrine B (II) (1) and several related hypotensive ester alkaloids (2, 3) made feasible the systematic alteration of the molecules. On the clinical side, recent work has demonstrated significant differences between protoveratrine A and protoveratrine B when the substances are administered orally, particularly with regard to hypotensive-emetic relationships (4–6). Since protoveratrines A and B differ only in the nature of the ester moiety at C_3, it was deemed desirable that new protoverine (III) (7) esters, which differ from the protoveratrines solely in the nature of the acid residues, should be prepared and subjected to pharmacological evaluation.

In our previous studies the compounds examined were protoverine derivatives with varying substitution at positions 3, 4, 6, 7, 14, 15, and 16 (8–10). The results supported the following generalizations (*a*) esterification at position 16 with acetate or isobutyrate is accompanied by a profound loss in activity, (*b*) esterification at positions 3 and 15 is required for high activity, (*c*) esterification at position 15 with a branched chain acid is advantageous, (*d*) the ester grouping at position 3 need not be branched, (*e*) positions 6 and 7 need not be esterified for good activity, (*f*) esterification at position 7 with a branched chain acid may be disadvantageous, (*g*) oxidation of the alcohol group at position 16 to a ketone group is accompanied by a loss in activity, (*h*) acetonide formation at positions 14 and 15 is accompanied by a profound loss in activity, (*i*) esterification at position 4 may be disadvantageous, and (*j*) considerable alteration can be made in the structure of the ester affixed at 3 without greatly altering hypotensive potency.

In view of the aforementioned facts that minor differences in the ester affixed at 3 significantly affect the hypotensive-emetic ratio of the protoveratrines and that changes in the structure of the ester at C_3 do not greatly alter hypotensive potency, it was deemed desirable that additional compounds which differ from each other only in the nature of the ester at C_{15} should be prepared and subjected to pharmacological evaluation. The present report details the synthesis and preliminary pharmacological evaluation of three new analogs of the protoveratrines. In the new analogs, the isobutyrate residue, previously demonstrated to be an effective substitute for the naturally-occurring substituted α-methylbutyrate residues (8), is affixed at C_3. Treatment of protoverine (III) with acetone and hydrochloric acid yielded the 14, 15-acetonide (IV), and acetylation then gave protoverine 14, 15-acetonide 3, 6, 16-triacetate (V). Upon treatment with sodium borohydride in *t*-butanol, protoverine 14, 15-acetonide 6, 16-diacetate (VIII) was obtained (7,11). Acylation with isobutyryl chloride in pyridine afforded the 14, 15-acetonide 6, 16-diacetate 3-isobutyrate (VII), and dilute acid gave protoverine 6, 16-diacetate 3-isobutyrate (VI). The latter triester served as the key intermediate for all the further synthesis. Partial acylation with the appropriate acid halide introduced the desired new ester at position 15 (IX, R = tosyl; XII, R = tigloyl; XV, R = nicotinyl). Treatment with acetic anhydride gave

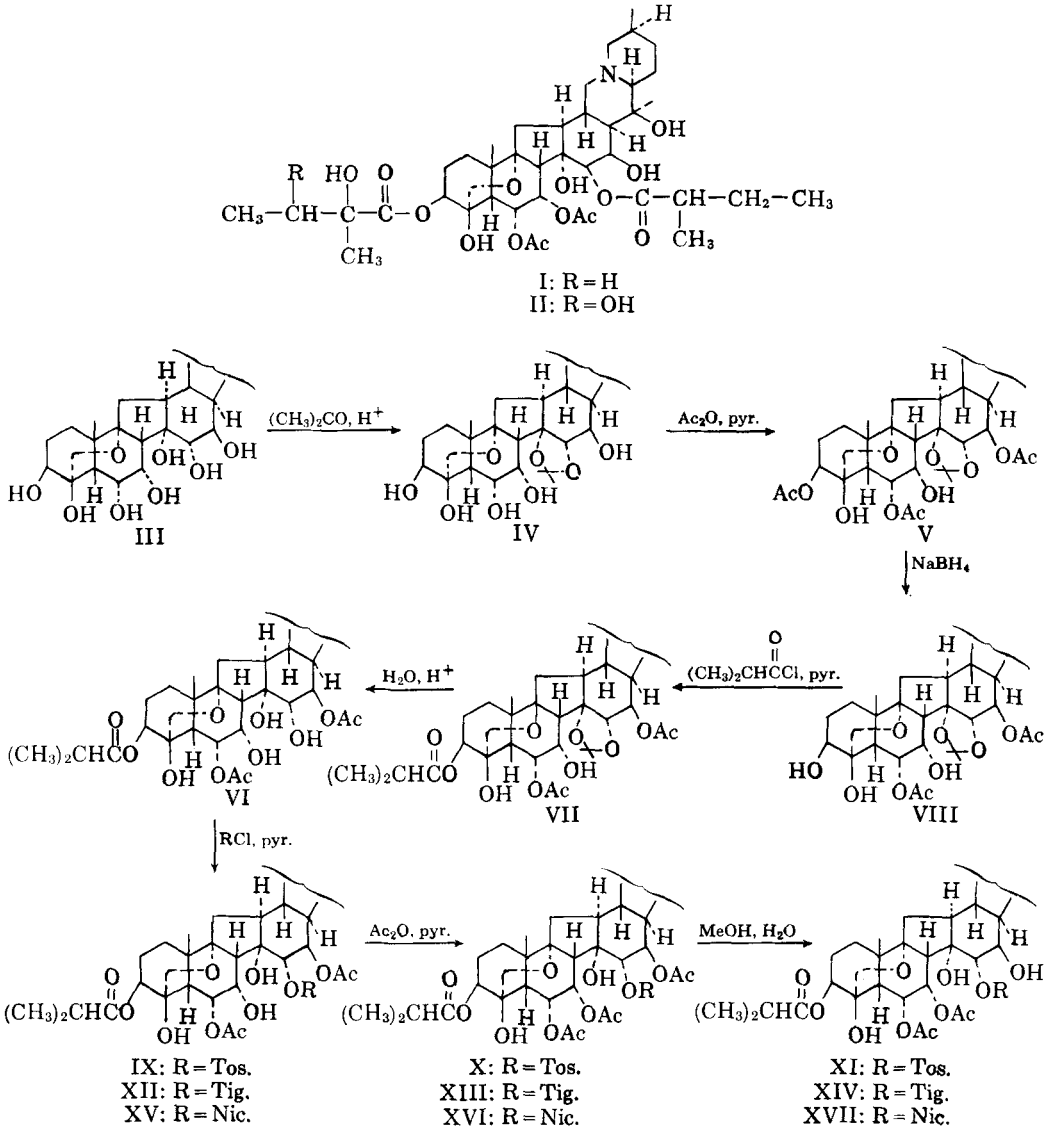
Received February 19, 1962, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison, and the Pharmacology Laboratories, Pitman-Moore Co., Division of the Dow Chemical Co., Indianapolis, Ind.

Accepted for publication March 27, 1962.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962, as part of a symposium entitled "Biologically Active Natural Products and Derived Synthetic Drugs."

Part XLIX in the series: Kupchan, S. M., Gruenfeld, N., and Katsui, N., *J. Med. Pharm. Chem.*, **5**, 690(1962).

The investigation at the University of Wisconsin was supported in part by research grants from the Wisconsin Alumni Research Foundation and Pitman-Moore Co.



the 3, 6, 7, 15, 16-pentaesters X, XIII, and XVI, and methanolysis selectively removed the 16-acetate to yield the desired 3, 6, 7, 15-tetraesters XI, XIV, and XVII.

The three new protoveratrine analogs XI, XIV and XVII have been investigated for hypotensive activity in the unanesthetized dog and the results are presented in Table I. Adult mongrel dogs unselected as to sex were employed in all experiments described. The femoral artery of the unanesthetized dog was catheterized using locally administered procaine for anesthesia. A mercury manometer and smoked kymogram were used to record mean blood pressure. The dog was placed in an upright position. Test drugs were administered intravenously as freshly prepared aqueous solutions, usually acidic. The effects of these drugs on systemic blood pressure and on emesis production were determined and the results are summarized in Table I. Responses to protoveratrine A and B, and to protoverine 3, 15-diisobutyrate 6,7-diacetate (8) are included for comparison. The 15-isobutyrate was moderately potent as a hypotensive agent, although the activity was appreciably reduced as compared to the protoveratrines. The 15-tiglate (XIV) retained activity, but the order of activity was considerably lower than that of the 15-isobutyrate. When the ester affixed at position 15 was tosylate (XI) or nicotinate (XVII), there was almost complete loss of activity within the doses tested.

It is apparent from the data reported herein that alterations in the structure of the ester moiety affixed at position 15 greatly affect the hypotensive potency. The situation contrasts

TABLE I.—EMETIC AND HYPOTENSIVE ACTIVITIES OF PROTOVERINE DERIVATIVES IN UNANESTHETIZED DOGS

	Mcg./Kg., i.v.	Mean Blood Pressure: No. Dogs	Change, %	Duration, min.	Emesis: No. Vomiting / No. Tested	Onset, min.
Protoveratrine A (I)	1	5	−22	39	1/5	87
	2	3	−37	75	2/3	24, 61
Protoveratrine B (II)	2	6	−33	25	4/6	2, 21
						95, 95
Protoverine 3,15-diisobutyrate 6,7-diacetate	2	2	0	0	0/2	
	4	3	−67	17	0/3	
	8	4	−62	8	0/4	
	16	2	−77	>30	2/2	17, 112
Protoverine 3-isobutyrate 6,7-diacetate 15-tosylate (XI)	4	1	0	0	0/1	
	16	1	−12	55	0/1	
	32	2	−37	1	0/2	
	64	2	−74	1	0/2	
Protoverine 3-isobutyrate 6,7-diacetate 15-tiglate (XIV)	2	3	0	0	0/2	
	64	2	−54	>40	1/2	4
	128	2	−71	40	1/2	1
Protoverine 3-isobutyrate 6,7-diacetate 15-nicotinate (XVII)	4	1	+43	5	0/1	
	64	1	0	...	0/1	
	128	2	−42	1	0/2	

with that of position 3-analogs of the protoveratrines, among which considerable alterations can be made in the structure of the ester without greatly altering hypotensive potency (10).

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology on samples dried under reduced pressure at 110°.

Protoverine 14,15-Acetonide 6,16-Diacetate (VIII).—A mixture of 16.2 Gm. of protoverine 14, 15-acetonide, 3, 6, 16-triacetate (V) (7), 1.52 L. of *t*-butanol and 3.8 Gm. of sodium borohydride was stirred at room temperature for three hours. A 70 ml. quantity of water was added, the solution was allowed to stand for 15 minutes, acidified with 105 ml. of acetic acid and evaporated under reduced pressure. The residue was taken up in water, made alkaline with dilute ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield a resin which was chromatographed on 272 Gm. of Merck acid-washed alumina. The column yielded to methanol-chloroform (0.3–1.7%) a resin which was crystallized from acetone as prisms, (8.74 Gm.), m.p. 236–238° decompn. (11). A paper chromatogram[1] indicated that the material was homogeneous.

Protoverine 14, 15-Acetonide 3-Isobutyrate 6, 16-Diacetate (VII).—A 13.73 Gm. quantity of protoverine 14, 15-acetonide 6, 16-diacetate (VIII) was dissolved in 135 ml. of pyridine, cooled in an ice bath, and isobutyryl chloride (6.66 ml., *ca.* 3 mole equivalents) was slowly added with stirring. The flask was protected from moisture with a calcium chloride tube. After completion of the acid chloride addition, the reaction mixture was allowed to stand at room temperature for 24 hours. The solution was treated with ice water, made alkaline with dilute ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. To remove all of the pyridine, the residue was repeatedly dissolved in benzene and evaporated to dryness, whereupon a yellow resin weighing 14.42 Gm. was obtained. The material was chromatographed on 225 Gm. of Merck acid-washed alumina and yielded to chloroform-benzene (65–80% chloroform), to chloroform, and to methanol-chloroform (0.3–3.3% methanol) a material which was crystallized from ether as prisms, (10.96 Gm.), m.p. 173–178° decompn. A paper chromatogram[1] indicated that the material was homogeneous. A sample was twice recrystallized for analysis from ether to yield prisms, m.p. 175–177° decompn., $[\alpha]_D^{25}$ + 28° (c 1.06, pyr.).

Anal.—Calcd. for $C_{38}H_{57}NO_{12} \cdot H_2O$: C, 61.87; H, 8.00. Found: C, 61.67, H, 7.64.

Protoverine 3-Isobutyrate 6, 16-Diacetate (VI).—A 6.20 Gm. quantity of protoverine 14, 15-acetonide 6, 16-diacetate (VII) was dissolved in 160 ml. of dilute hydrochloric acid (1:4) and the solution was allowed to stand at room temperature for 40 minutes. The reaction mixture was treated with ice, made alkaline with dilute ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield an almost colorless residue. Chromatography on 75 Gm. of Merck acid-washed alumina yielded to chloroform-benzene (35–80%), to chloroform, and to methanol-chloroform (0.5–1.5%), a resin which was crystallized from acetone as rods (5.02 Gm.), m.p. 221–224° decompn. A paper chromatogram[1] indicated that the material was homogeneous. A sample was twice recrystallized from acetone as rods, m.p. 225–226° decompn., $[\alpha]_D^{25}$ − 19° (c 1.15, pyr.).

Anal.—Calcd. for $C_{35}H_{53}NO_{12} \cdot {}^1/_2\ H_2O$: C, 61.04; H, 7.84. Found: C, 60.80; H, 7.48.

Protoverine 3-Isobutyrate 6,16-Diacetate 15-Tosylate (IX).—A 1.9 Gm. quantity of tosyl chloride was added portionwise to a solution containing 679 mg. of protoverine 3-isobutyrate 6, 16-diacetate (VI) in 6 ml. of pyridine cooled in an ice bath. The mixture was allowed to stand at room temperature for 24 hours. The reaction mixture was treated with ice, water, and dilute ammonium hydroxide to

[1] The paper chromatographic system was that of Levine and Fischbach (12): the solution was prepared by adding 1 ml. of formic acid to the separated solvent layer of the system *n*-butylacetate: *n*-butanol: water (10:25:10 ml.).

pH 8.5 and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was repeatedly dissolved in benzene and evaporated to dryness to remove pyridine traces. Crystallization of the residue from acetone yielded prisms weighing 506 mg., m.p. 224–226° decompn. Paper chromatographic analysis[2] indicated the material was homogeneous. The product was twice recrystallized from acetone to yield prisms, m.p. 230–231° decompn., $[\alpha]_D^{26}$ − 5° (c 1.37 pyr.).

Anal.—Calcd. for $C_{42}H_{59}NO_{14}S$: C, 60.50; H, 7.08; S., 3.84. Found: C, 60.73; H, 7.01; S, 3.85.

Protoverine 3-Isobutyrate 6,7,16-Triacetate 15-Tosylate (X).—A solution containing 416 mg. of protoverine 3-isobutyrate 6,16-diacetate 15-tosylate (IX) in 3 ml. of pyridine was treated with 12 ml. of acetic anhydride and heated in a water bath at 80° for 2.5 hours. The reaction mixture was treated with ice, water, and dilute ammonium hydroxide to pH 8.5 and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residual resin was crystallized from acetone as prisms, (357 mg.), m.p. 245–247° decompn. A paper chromatogram[2] indicated the homogeneity of the material. Recrystallization from acetone gave prisms, m.p. 245–247° decompn., $[\alpha]_D^{27}$ − 38° (c 1.95, pyr.).

Anal.—Calcd. for $C_{44}H_{61}NO_{15}S$: C, 60.34; H, 6.97; S, 3.65. Found: C, 60.31; H, 7.04; S, 3.44.

Protoverine 3-Isobutyrate 6,7-Diacetate 15-Tosylate (XI).—A solution containing 350 mg. of protoverine 3-isobutyrate 6,7,16-triacetate 15-tosylate (X) in 30 ml. of methanol was allowed to stand at room temperature for 64 hours. Evaporation to dryness left a colorless solid which was crystallized from acetone, to yield prisms, (202 mg.), m.p. 194–195° followed by decomposition at 235–237°. Paper chromatographic analysis[3] indicated the principal product was contaminated with components of lower R_f. Two recrystallizations from acetone yielded prisms weighing 99 mg., m.p. 199–200° followed by decomposition at 215°, $[\alpha]_D^{25}$ − 31° (c 1.14 pyr.), and this material was demonstrated to be homogeneous by paper chromatography.

Anal.—Calcd. for $C_{42}H_{59}NO_{14}S$: C, 60.50; H, 7.08; S, 3.84. Found: C, 60.35; H, 7.19; S, 3.80.

Protoverine 3-Isobutyrate 6,16-Diacetate 15-Tiglate (XII).—A 0.15 ml. quantity of tigloyl chloride was added dropwise to a solution containing 340 mg. of protoverine 3-isobutyrate 6,16-diacetate (VI) in 3.4 ml. of pyridine cooled in an ice bath. The mixture was allowed to stand at 0° for 1 hour and then at room temperature for 18 hours. Workup as described for IX gave a yellow resin which was chromatographed on 12.5 Gm. of Merck acid-washed alumina. The column yielded to chloroform and to methanol-chloroform (0.5–1.5%) a resin which was crystallized from acetone to yield plates: first crop, 155 mg., m.p. 229–230° decompn.; second crop, 29 mg., m.p. 225–226° decompn. Paper chromatography[2] indicated that the first crop was homogeneous and that the second crop was contaminated with a small proportion of a second substance. Recrystallization of a sample of the first crop from acetone yielded plates, m.p. 232–233° decompn., $[\alpha]_D^{27}$ + 13° (c 1.85, pyr.).

Anal.—Calcd. for $C_{40}H_{59}NO_{13}$: C, 63.06; H, 7.75. Found: C, 63.17; H, 7.81.

Protoverine 3-Isobutyrate 6,7,16-Triacetate 15-Tiglate (XIII).—A solution containing 304 mg. of protoverine 3-isobutyrate 6,16-diacetate 15-tiglate (XII) in 2.4 ml. of pyridine was treated with 1.0 ml. of acetic anhydride as described for X. Workup in the usual manner gave a resin which was crystallized from acetone as prisms, (214 mg.), m.p. 260–261° decompn. A second crop weighing 49 mg., m.p. 255–259° decompn., was also obtained. Both crops were shown to be homogeneous by paper chromatography[2]. A sample was twice recrystallized for analysis from acetone to yield prisms, m.p. 264–265° decompn., $[\alpha]_D^{28}$ − 22° (c 1.55, pyr.).

Anal.—Calcd. for $C_{42}H_{61}NO_{14}\cdot H_2O$: C, 61.38; H, 7.67. Found: C, 61.63; H, 7.65.

Protoverine 3-Isobutyrate 6,7-Diacetate 15-Tiglate (XIV).—A solution containing 545 mg. of protoverine 3-isobutyrate 6,7,16-triacetate 15-tiglate (XIII) in 55 ml. of methanol was allowed to stand at room temperature for 27 hours. Evaporation to dryness left a colorless resin which was chromatographed on 11 Gm. of Merck acid-washed alumina. The column yielded to chloroform a resin which was crystallized from ether as prisms (116 mg.). Recrystallization from ether gave prisms weighing 80 mg., m.p. 229–233°, $[\alpha]_D^{28}$ − 22° (c 1.05, pyr.), shown to be homogeneous by paper chromatography.[4]

Anal.—Calcd. for $C_{40}H_{59}NO_{13}$: C, 63.06; H, 7.75. Found: C, 63.06; H, 7.76.

Protoverine 3-Isobutyrate 6,16-Diacetate 15-Nicotinate (XV).—A 0.26 ml. quantity of nicotinyl chloride was added dropwise to a solution containing 679 mg. of protoverine 3-isobutyrate 6,16-diacetate (VI) in 6.8 ml. of pyridine cooled in ice water. After 17 hours at room temperature, the reaction mixture was worked up by the procedure described for IX to yield a resin from chloroform. Crystallization from acetone gave prisms weighing 520 mg., m.p. 200–204° decompn., and a second crop weighing 76 mg., m.p. 195–202° decompn. Both fractions were shown to be homogeneous by paper chromatography.[4] A sample was recrystallized for analysis from acetone to yield prisms, m.p. 200–205° decompn., $[\alpha]_D^{28}$ + 28° (c 1.53, pyr.).

Anal.—Calcd. for $C_{41}H_{56}N_2O_{13}\cdot H_2O$: C, 61.27; H, 7.23; N, 3.49. Found: C, 61.50; H, 6.92; N, 3.62.

Protoverine 3-Isobutyrate 6,7,16-Triacetate 15-Nicotinate (XVI).—A solution containing 770 mg. of protoverine 3-isobutyrate 6,16-diacetate 15-nicotinate (XV) in 6 ml. of pyridine was treated with 2.5 ml. of acetic anhydride as described for X. Workup in the usual manner gave a resin which was crystallized from acetone as prisms weighing 663 mg., m.p. 253–254° decompn. A sample was twice recrystallized from acetone to yield prisms, m.p. 256–257° decompn., $[\alpha]_D^{27}$ − 23° (c 1.79, pyr.).

Anal.—Calcd. for $C_{43}H_{58}N_2O_{14}$: C, 61.98; H, 7.02; N, 3.38. Found: C, 61.80; H, 7.02; N, 3.33.

Protoverine 3-Isobutyrate 6,7-Diacetate 15-Nicotinate (XVII).—A solution containing 346 mg. of

[2] The paper chromatographic system used was *n*-butyl acetate : *n*-butanol : formic acid (25 : 5 : 1 by volume) (12).

[3] The solvent system used was : *n*-butyl acetate : *n*-butanol : formic acid (100 : 1 : 5 by volume), saturated with water.

[4] The solvent system used was: 2,2,4-trimethylpentane: 4-methyl-2-pentanone: pyridine (10:5 : 1 by volume).

protoverine 3-isobutyrate 6,7,16-triacetate 15-nicotinate (XVI) in 57 ml. of methanol was allowed to stand at room temperature for 16 hours. Evaporation to dryness left a resin which resisted attempts at purification by column chromatography, but which yielded to preparative paper chromatography. The resin was dissolved in chloroform and applied to Whatman No. 4 paper (19 sheets, 6 × 18 inches) pretreated with phosphate buffer of pH 3.5. After development for 2.5 hours with the isooctane solvent system[4] and drying, a chloroform solution of Bromphenol Blue was sprayed over the papers as usual. The desired band was cut from the sheets and extracted with 2% methanol-chloroform in a Soxhlet extractor for 2 hours. The extract was shaken with dilute ammonium hydroxide to liberate the free base and remove the dye, and the chloroform solution was washed with water. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to yield a colorless resin weighing 205 mg., which was shown to be homogeneous by paper chromatography.[4] Crystallization from acetone-ether gave colorless prisms weighing 139 mg., m.p. 227–229° decompn. Recrystallization gave prisms, m.p. 229–230° decompn., $[\alpha]_D^{27}$ − 1° (c 1.08, pyr.).

Anal.—Calcd. for $C_{41}H_{56}N_2O_{13} \cdot 1/2\ H_2O$: C, 62.04; H, 7.18; N, 3.53. Found: C, 61.89; H, 7.11; N, 3.84.

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Veratrum Alkaloids LI

Hypotensive-Emetic Relationships in the Unanesthetized Dog Among Analogs of the Protoveratrines

By LAWRENCE C. WEAVER†, W. RALPH JONES†, and S. MORRIS KUPCHAN

A study aimed at elucidation of the relationship between hypotensive and emetic activities in the unanesthetized dog among synthetic analogs of the protoveratrines is reported. Preliminary data indicate that a partial dissociation of hypotensive and emetic activities is demonstrable.

VERATRUM and related plants have been used for medicinal purposes for hundreds of years. Galenical preparations were used in the Middle Ages for purposes of sorcery and mystical rites. Subsequently, the crude extracts have been used in the treatment of fevers, as local counterirritants in neuralgia, as cardiac tonics, as emetics, as crow poisons, and as insecticides (1, 2). The use of veratrum in the control of hypertension, at least in the United States, dates from the report of Baker in 1859 (3). Several attempts were made to introduce the use of veratrum extracts into medical practice during the second half of the nineteenth century, but these attempts were unsuccessful. The treatments during this period continued to employ crude extracts containing many alkaloids. The results achieved with these crude extracts were erratic and the treatments fell into disrepute. Some 50 years later, during the late 1930's, purified alkaloidal preparations responsible for the hypotensive activity of veratrum became available for the first time. Poethke in Germany (4) and Jacobs and Craig in the United States (5) improved the extraction and purification procedures and made available the first crystalline powerfully hypotensive alkaloid preparation, protoveratrine. Careful pharmacological investigation of the crystalline preparations, spearheaded by Krayer and his associates, demonstrated that the drugs were suitable for clinical trials (6). These clinical

Received March 12, 1962, from the Biomedical Research Department, Pitman-Moore Co., Division of the Dow Chemical Co., Indianapolis, Ind., and the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.
Accepted for publication May 14, 1962.
† Pitman-Moore Co., Indianapolis, Ind.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962, as part of the Symposium entitled "Biologically-Active Natural Products and Derived Synthetic Drugs."
Part L in the series: Kupchan, S. M., Fujita, E., Grivas, J. C., and Weaver, L. C., THIS JOURNAL, **51**, 1140(1962).

protoverine 3-isobutyrate 6,7,16-triacetate 15-nicotinate (XVI) in 57 ml. of methanol was allowed to stand at room temperature for 16 hours. Evaporation to dryness left a resin which resisted attempts at purification by column chromatography, but which yielded to preparative paper chromatography. The resin was dissolved in chloroform and applied to Whatman No. 4 paper (19 sheets, 6 × 18 inches) pretreated with phosphate buffer of pH 3.5. After development for 2.5 hours with the isooctane solvent system[4] and drying, a chloroform solution of Bromphenol Blue was sprayed over the papers as usual. The desired band was cut from the sheets and extracted with 2% methanol-chloroform in a Soxhlet extractor for 2 hours. The extract was shaken with dilute ammonium hydroxide to liberate the free base and remove the dye, and the chloroform solution was washed with water. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to yield a colorless resin weighing 205 mg., which was shown to be homogeneous by paper chromatography.[4] Crystallization from acetone-ether gave colorless prisms weighing 139 mg., m.p. 227–229° decompn. Recrystallization gave prisms, m.p. 229–230° decompn., $[\alpha]_D^{27}$ − 1° (c 1.08, pyr.).

Anal.—Calcd. for $C_{41}H_{56}N_2O_{13} \cdot 1/2\ H_2O$: C, 62.04; H, 7.18; N, 3.53. Found: C, 61.89; H, 7.11; N, 3.84.

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Veratrum Alkaloids LI

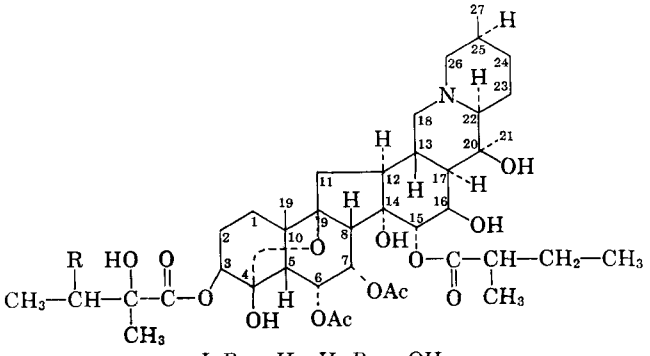
Hypotensive-Emetic Relationships in the Unanesthetized Dog Among Analogs of the Protoveratrines

By LAWRENCE C. WEAVER†, W. RALPH JONES†, and S. MORRIS KUPCHAN

A study aimed at elucidation of the relationship between hypotensive and emetic activities in the unanesthetized dog among synthetic analogs of the protoveratrines is reported. Preliminary data indicate that a partial dissociation of hypotensive and emetic activities is demonstrable.

VERATRUM and related plants have been used for medicinal purposes for hundreds of years. Galenical preparations were used in the Middle Ages for purposes of sorcery and mystical rites. Subsequently, the crude extracts have been used in the treatment of fevers, as local counterirritants in neuralgia, as cardiac tonics, as emetics, as crow poisons, and as insecticides (1, 2). The use of veratrum in the control of hypertension, at least in the United States, dates from the report of Baker in 1859 (3). Several attempts were made to introduce the use of veratrum extracts into medical practice during the second half of the nineteenth century, but these attempts were unsuccessful. The treatments during this period continued to employ crude extracts containing many alkaloids. The results achieved with these crude extracts were erratic and the treatments fell into disrepute. Some 50 years later, during the late 1930's, purified alkaloidal preparations responsible for the hypotensive activity of veratrum became available for the first time. Poethke in Germany (4) and Jacobs and Craig in the United States (5) improved the extraction and purification procedures and made available the first crystalline powerfully hypotensive alkaloid preparation, protoveratrine. Careful pharmacological investigation of the crystalline preparations, spearheaded by Krayer and his associates, demonstrated that the drugs were suitable for clinical trials (6). These clinical

Received March 12, 1962, from the Biomedical Research Department, Pitman-Moore Co., Division of the Dow Chemical Co., Indianapolis, Ind., and the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.
Accepted for publication May 14, 1962.
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Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962, as part of the Symposium entitled "Biologically-Active Natural Products and Derived Synthetic Drugs."
Part L in the series: Kupchan, S. M., Fujita, E., Grivas, J. C., and Weaver, L. C., THIS JOURNAL, **51**, 1140(1962).



I, R = H II, R = OH

trials were followed by introduction of protoveratrine and related preparations into clinical use in the treatment of certain types of hypertension (7–9). The limiting factor in the clinical use of these drugs has been the narrow dosage range between hypotensive and emetic effects.

In 1952–1953, four groups reported almost simultaneously that protoveratrine was not a homogeneous entity, but a mixture of two closely related ester alkaloids, protoveratrine A and protoveratrine B (10–13). Subsequent clinical studies have established significant differences between protoveratrine A and protoveratrine B when the substances are administered orally (14–16). Protoveratrine A is a potent hypotensive agent with a narrow therapeutic dosage range. Protoveratrine B, on the other hand, is inactive orally in doses several times the hypotensive doses of protoveratrine A. However, studies of divided doses of more than 10 mg. a day have indicated that protoveratrine B has strong hypotensive activity, which may be prolonged and not accompanied by emetic effects (14–16).

Structural studies culminated in 1960 with elucidation of the complete structures and configurations of the alkamine protoverine and of the tetraesters protoveratrine A (I) and protoveratrine B (II) (17, 18). It is evident that the structures of the two protoveratrines are exceedingly similar, and, indeed, differ only in the nature of the acid moiety of the ester at C_3. In view of the aforementioned pronounced difference in activity which accompanies this minor difference in structure, it was deemed desirable that new analogs of the protoveratrines should be prepared and subjected to pharmacological evaluation. It was hoped that such a study might point the way to the preparation of new protoverine derivatives with more favorable hypotensive-emetic ratios.

To study the relationship between hypotensive activity and structural isomerism among polyesters of protoverine, the first phase of the program involved the synthesis of all the possible protoverine 3,6,7,15-tetraesters which contain one, two, or three isobutyryl residues, and in which the remaining acyl groups are acetyl (19). Pharmacological evaluation of the synthetic esters in *anesthetized* dogs led to formulation of the following generalizations: (*a*) esterification at position 16 with acetate or isobutyrate is accompanied by a profound loss in activity, (*b*) esterification at positions 3 and 15 is required for high activity, (*c*) esterification at position 15 with a branched-chain acid is advantageous, (*d*) the ester grouping at position 3 need not be branched, (*e*) positions 6 and 7 need not be esterified, and (*f*) esterification at position 7 with a branched-chain acid may be disadvantageous.

Our second study involved a broader group of structural variants, and the results supplemented and extended earlier generalizations (20). New relationships which evolved from the second study were: (*g*) oxidation of the alcohol group at position 16 to a ketone group is accompanied by a loss in activity, (*h*) acetonide formation at positions 14 and 15 is accompanied by a profound loss in activity, and (*i*) esterification at position 4 may be disadvantageous.

In our third study, 25 new protoverine tetraesters which differ from the protoveratrines solely in the nature of the acid residue affixed at position 3 were described (21). Pharmacological evaluation in anesthetized dogs indicated that considerable alterations can be made in the structure of the ester affixed at position 3 without greatly altering hypotensive potency.

The most recent study involved the synthesis and evaluation of a series of protoverine tetraesters which differ from each other only in the nature of the acid residue at position 15. Pharmacological evaluation of the new protoverine tetraesters showed substantial diminution in hypotensive activity when compared to the protoveratrines (22).

METHODS

The present report is concerned with an evaluation of selected active compounds in the *unanesthetized* dog. Adult mongrel dogs, unselected as to sex, were used for these studies. Mean blood pressure was recorded on a smoked kymogram from the catheterized femoral artery of the dog maintained in a standing position. All drugs were administered rapidly as aqueous solutions, usually acidic, by the intravenous route. The dogs were observed for approximately 2 hours to determine duration of changes in systemic blood pressure and signs of emesis. It should be emphasized that only one dose of one drug was given to any dog. Thus, tolerance or cumulation were not variables. When evaluating compound effects, one should consider hypotensive degree and duration, as well as the presence or absence of emesis.

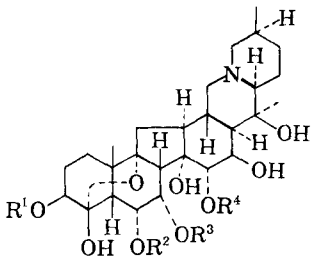
RESULTS AND DISCUSSION

Ten of the synthetic protoverine tetraesters of the "first study" showed activity in anesthetized dogs in dosages less than 128 mcg./Kg. i.v. (19). These compounds have been studied in the unanesthetized dog and the results are presented in Table I. The compounds are numbered as in ref. 19 in order to make comparisons with data in anesthetized dogs convenient. Generally, the compounds at equivalent dosages had less hypotensive effect than was observed in the anesthetized dog. This is not unexpected, since a barbiturate was the anesthetic agent used in the early studies. Only compound VI had a margin between hypotensive and emetic effects greater than protoveratrine A or B; it is questionable whether this margin is significant. It should be pointed out that when emesis occurred, the duration of the hypotensive response as recorded was frequently decreased because of the changes in blood pressure secondary to this emesis. The hypotensive-emetic ratio of the other compounds appeared similar to those of the protoveratrines.

None of the compounds from the "second study" exhibited sufficient activity to qualify them for investigation in unanesthetized dogs.

All of the 26 new protoveratrine analogs of the

TABLE I.—HYPOTENSIVE-EMETIC RELATIONSHIPS OF PROTOVERINE DERIVATIVES IN UNANESTHETIZED DOGS

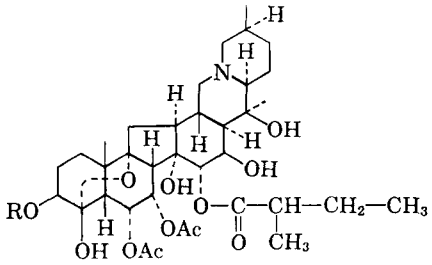


Compound No.	R^{1a}	R^2	R^3	R^4	Dose, mcg./Kg. i.v.	No. Dogs	Blood Pressure—Change, %	Blood Pressure—Duration, min.	Emesis—No. Vomiting/No. Tested	Emesis—Onset, min.
III	B	B	H	B	32	3	−53	8	0/4	
					64	3	−62	28	1/5	11
					128	2	−59	1	1/2	30
VI	A	B	A	B	16	2	−41	75	0/6	
					32	2	−60	225	3/6	5, 5, 10
VII	B	B	A	B	32	3	−41	19	3/5	1, 5, 7
					64	2	...	...	2/2	6, 11
XII	A	A	B	B	32	3	−46	3	1/3	97
XIII	A	A	A	B	4	3	−85	2	1/3	28
					8	1	−58	2	1/1	98
XVIII	A	B	A	A	128	3	−15	2	1/3	16
XXXIX	B	A	B	B	64	3	−44	22	0/3	
					128	4	−77	32	1/3	94
					256	1	−59	26	1/1	1
XL	B	A	A	B	4	3	−67	13	0/3	
					8	4	−62	8	0/4	
					16	2	−77	>30	2/2	17, 112
XLVIII	B	A	H	B	2	3	−50	8	0/3	
					4	4	−75	75	3/5	7, 55, 110
XLIX	B	H	H	B	4	3	−47	27	0/3	
					8	3	−71	9	2/3	3, 114
Protoveratrine	A				1	5	−22	39	1/5	87
					2	3	−37	75	2/3	24, 61
Protoveratrine	B				1	6	−33	25	4/6	2, 21, 95, 95

[a] A, acetate; B, isobutyrate; H, hydrogen.

"third study," *i.e.*, the compounds differing from the protoveratrines solely in the nature of the ester at position 3, showed significant hypotensive activity in anesthetized dogs at dosages of 128 mcg./Kg. i.v. or less (21). The results obtained with the latter compounds when administered to unanesthetized dogs is presented in Table II. The compounds are numbered as in ref. 21 to make comparisons with data in anesthetized dogs convenient. Further, an "a" is added (*e.g.*, Ia) to prevent confusion with compounds mentioned earlier in this report. The data for escholerine (IIIa) are included, as well. As was noted above in comparing data obtained from anesthetized and unanesthetized dogs, differences were usually in the direction of lesser activity in the unanesthetized dog. Test compounds were less active than protoveratrine A and B in most cases. Exceptions were compounds IVa, VIa, XIa, XVIIIa, XXIIa, and XXVIa. Increased hypotensive-emetic ratios or margins of safety appeared to be present with compounds IXa and XIVa. Thus, preliminary data indicate that a partial dissociation of hypotensive and emetic activities among synthetic analogs of the protoveratrines is demonstrable in the unanesthetized dog. Compounds XXa, XXIVa, and XXIXa failed to show hypotensive activity at the doses tested, although emesis was evoked. The latter results may be considered to constitute support for the thesis that hypotensive-emetic ratios can be altered. Several of the new analogs will be investigated in human hypertensives to determine whether the improved margin is demonstrable in man as well.

TABLE II.—HYPOTENSIVE-EMETIC RELATIONSHIPS OF DESATRINE DERIVATIVES IN UNANESTHETIZED DOGS



Compound No.	R	Dose, mcg./Kg. i.v.	No. Dose	Blood Pressure: Change, %	Blood Pressure: Duration, min.	Emesis: No. Vomiting/ No. Tested	Emesis: Onset, min.
IIIa	Angelate	4	7	−49	>61	4/7	2, 3, 7, 15
		16	2	...	...	2/2	...
IVa	Tiglate	2	2	−33	31	0/2	
		8	4	−49	>64	1/4	4
Va	Acetate	16	2	−62	16	1/2	16
		32	2	...	...	2/2	...
VIa	Isobutyrate	1	2	−65	6	1/2	50
		2	2	−68	68	1/2	15
		4	2	−51	171	2/2	2, 4
		8	2	...	...	1/2	...
VIIa	Benzoate	8	2	−57	4	2/2	2, 16
VIIIa	3′,4′-Methylene dioxybenzoate	8	2	−57	25	1/2	16
IXa	Trichloroacetate	4	1	14	30	0/1	
		8	2	−12	60	0/2	
		16	4	−45	12	0/4	
		32	3	−68	12	2/3	5, 11
Xa	Chloroacetate	2	1	−39	5	1/1	105
		4	2	−74	5	0/2	
		8	2	−52	21	1/2	2
XIa	Nicotinate	1	3	−19	7	1/3	19
		2	4	−57	37	1/4	11
		4	4	−59	47	4/4	4, 9, 13, 24
		8	2	−75	>60	2/2	3, 5
XIIa	4′-Nitrobenzoate	4	2	−39	56	0/2	
		8	3	−39	6	0/3	
		16	6	−64	>53	3/6	28, 48, 70
XIIIa	4′-Nitrohexanoate	4	3	−63	11	0/3	
		8	2	−66	23	1/2	8
XIVa	Tosylate	8	2	−21	34	0/2	
		16	2	−20	88	0/2	
		32	1	−45	120	0/1	
XVa	Diethylphosphate	32	3	−41	3	0/3	
		64	2	−73	17	0/2	
		128	2	−45	40	2/2	1, 1

TABLE II (*continued*)

Compound No.	R	Dose, mcg./Kg. i.v.	No. Dogs	Blood Pressure		Emesis	
				Change, %	Duration, min.	No. Vomiting/ No. Tested	Onset, min.
XVIa	N,N-Diethylamino-acetate	4	3	0	0	0/2	
		8	2	−61	19	1/3	44
		16	1	−34	4	0/1	
		32	3	−62	38	2/3	1, 3
XVIIa	3′-Acetoxy-2′-hydroxy-2′-methylbutyrate	4	3	−34	95	2/3	60, 100
		16	2	...	...	2/2	...
XVIIIa	3′-Isobutyroxy-2′-hydroxy-2′-methylbutyrate	4	4	−26	3	2/4	4, 24
		16	1	−72	2	lethal	
XIXa	3′-Tigloxy-2′-hydroxy-2′-methylbutyrate	8	3	−22/22	29	1/3	11
		32	1	−54	31	1/1	10
XXa	3′-Benzoxy-2′-hydroxy-2′-methylbutyrate	4	2	0	0	0/2	
		8	1	0	0	0/1	
		16	1	0	0	1/1	26
XXIa	3′-(4″-Nitrobenzoxy)-2′-hydroxy-2′-methylbutyrate	16	2	−19	40	1/2	7
XXIIa	3′-Nicotinoxy-2′-hydroxy-2′-methylbutyrate	4	7	−12	30	3/7	18, 19, 30
		8	8	−31	30	6/8	10, 14, 17 20, 20, 27
		16	2	−62	>45	1/1	4 (1 died)
XXIIIa	3′-Chloroacetoxy-2′-hydroxy-2′-methylbutyrate	8	3	−50	48	1/3	20
		16	4 2	−50	20	1/4	30
XXIVa	3′-Tosyloxy-2′-hydroxy-2′-methylbutyrate	4	2	10	9	1/2	18
		8	2	...	...	2/2	...
		16	2	...	...	2/2	...
XXVa	2′,3′-Epoxy-2′-methylbutyrate	8	2	−34	22	0/2	
		16	2	...	...	2/2	...
XXVIa	3′-Chloro-2′-hydroxy-2′-methylbutyrate	2	3	−25	30	1/3	120
		4	4	−55	50	2/4	3, 11
XXVIIa	3′-Hydroxy-2′-fluoro-2′-methylbutyrate	4	3	−34	>16	3/3	6, 10, 31
XXVIIIa	3′-N,N-Diethylamino-acetoxy-2′-hydroxy-2′-methylbutyrate	4	1	−21	12	0/1	
		8	4	−27	32	2/4	7, 67
		16	1	−100	...	lethal	
XXIXa	3′-(4″-Aminobenzoxy)-2′-hydroxy-2′-methylbutyrate	4	1	0	0	0/1	
		8	2	8	18	0/2	
		16	4	21	5	2/4	8, 15

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Probability and Complexation: A New Approach

By LLOYD KENNON and KUO-SIN CHEN†

A method is proposed which considers the ability of the ionic atmosphere surrounding a drug in solution to form "probability complexes" with the drug. These complexes are detectable both mathematically and physically. The method is described and illustrated with a study of the behavior of three solutions of phenothiazine derivatives with saccharin.

WHAT is a complex? For about the last ten years this question has been asked, with particular interest being expressed in the components of pharmaceutical solutions. When one attempts to answer this question with various mechanistic concepts, it is found that exact quantitation and definition may be somewhat elusive. However, an explanation which is unencumbered by mechanisms and, hence, probably completely uncontroversial is this: A and B are complexed when there are more A species around and/or closer to B species than a random distribution of A's and B's would bring about. It is such a concept which this communication seeks to explore and discuss.

THEORY AND BACKGROUND

Although the ordered state of crystalline solids, with their units locked into symmetrical and repeating patterns, and the disordered, randomized state of gas molecules have received much attention and investigation and are relatively well understood, the amorphous solid state (including glasses) and liquids have not been so explicitly characterized. At one time the latter two states had in general been considered to be somewhat similar, in that they are both structureless and amorphous. It is now well-known that both dissolved solutes and liquid solvents *per se* behave as if they have at least transitory structures.

The concept of incomplete dissociation, even with complete ionization resulting in nondistinguishable, truly unionized molecules and associated ion-pairs, is well accepted with regard to solutes. Fuoss and Kraus (1) have postulated the existence of ion triplets in addition to the pairs. The Debye-Hückel-Onsager treatments of the theory of electrolytic solutions which were made during their development of the interionic attraction concepts recognize that each ion is surrounded by its own ionic atmosphere of opposite charge. These phenomena, of course, more readily exist in solvents having lower dielectric constants. Also, Brady (2, 3) has demonstrated by X-ray measurements that ionic solutions have structure, *i.e.*, a large degree of local order. Other authorities such as Bailar (4) and Martell and Calvin (5) have drawn attention to chelation processes, coordination compounds, complex inorganic compounds such as amines, hydrates, and double salts, and olation and oxolation. Olation, whereby metal atoms are linked together by OH bridges, can cause the formation of polymers which may reach colloidal size when the olation process does not terminate early. Oxolation, conversion of *ol* groups to *oxo* groups by proton loss, can function similarly. Schlenk and Sand (6) have shown that even clathrates or inclusion compounds can exist in the liquid phase.

Solvents themselves are also capable of sustaining a structure, at least instantaneously. This has been recognized by workers such as Bernal (7–9). Even though the neighbors of a liquid's molecules appear to lie at apexes of various kinds of polyhedra, the highly condensed nature of liquids and their lack of long range order has made the problem of constructing realistic geometric models for mathematical analysis very difficult.

It is known that complexation concepts have explained some of the behavior noted in pharmaceutical solutions. For example Higuchi and Lachman (10) in their first paper on the inhibition of ester hydrolysis have shown that benzocaine does not hydrolyze in solution when it is present as a complex with caffeine. This, and similar observations along with the structure-in-liquids concepts, leads one to believe that another pharmaceutical observation may be explicable and studied as outlined in this paper. The observation is this: Often in pharmaceutical development it appears that different salts of the same compound exhibit not only different solubility characteristics but also differing stability properties. As a literature example we may cite the work of Macek (11) and Bird and Shelton (12) who noted differences in the stabilities of the hydrochloride and nitrate salts of thiamine.

These and similar results found in the course of pharmaceutical development work are somewhat surprising, because at any given pH the same fractions of the possible drug species exist in solution regardless of the acid salt used. Of course, some cases are not surprising and are more readily understood. Epinephrine fumarate or its equivalent has been shown by Romanian workers (13) to be more stable than epinephrine hydrochloride; a British patent (14) was issued to the Rhone-Poulenc Corp. on the use of chlorpromazine fumarate (or maleate, citraconate, itaconate, *etc.*) as preferred to the hydrochloride. These and similar cases may be explained by noting that an oxidizable olefinic linkage has been introduced and would be expected to impart some antioxidant properties. In fact, the Lindsey-Maxwell process (15) for stabilizing edible fatty oils is similar in that citric acid is added

Received March 9, 1962, from the Products Division, Bristol-Myers Co., Hillside, N. J.
Accepted for publication June 8, 1962.
Contribution from Smith Kline and French Laboratories, Philadelphia 1, Pa.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
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to the oil and then deliberately decomposed to form unsaturated, easily oxidized acids.

The thesis of this paper is that the surprising results mentioned above are due to the fact that the ionic environment *per se* plays a decisive role in the stability picture. This is true because the ions form a protective molecular overcoat—a loose ionic atmosphere complex—which is detectable both mathematically and physically and which may be reasonably termed a "probability complex." Thus, a probability complex mathematically just approaches the definition of a complex mentioned in the introduction; because of its physically demonstrable existence, we may say it does fit the definition.

Formulation work done in our laboratories by Gulesich and Marlino (16) showed that the presence of sodium saccharin in some chlorpromazine hydrochloride[1] ampul and syrup formulations surprisingly enhanced the light stability of the drug. Subsequent work indicated that saccharin also improved the stability to light of other phenothiazine derivatives such as prochlorperazine ethanedisulfonate[1] and trifluoperazine dihydrochloride.[1] These observations also supported the view that certain ions in solution may affect the stability of drugs. The aim of the experimental work was to show the existence of, isolate, and identify if possible the postulated probability complexes. The next section describes the probability method as part of the experimental work.

EXPERIMENTAL

Chlorpromazine and Saccharin.—The initial work was aimed at forcing the saccharin complexes out of solution. This was done by determining the maximum concentrations at which the phenothiazine derivative and sodium saccharin together would form a clear solution. The following illustration of the procedure will use chlorpromazine as the first example. It was found that the maximum permitted concentrations of the two components were 0.00625 *M* chlorpromazine hydrochloride and 0.00332 *M* sodium saccharin; this is a chlorpromazine: saccharin ratio of 1.88:1.00. The phenothiazine must be surrounded by both Cl and saccharin (S) species. At S = 1, Cl = 1.88, so the total possibilities of this (mutually exclusive) situation = 2.88. Therefore, the probability of the drug being in the saccharin form = Ps = 1/2.88 = 0.348; the probability of it being in the Cl form = PCl = 1.88/2.88 = 0.653. The sum of these two is 1.00 as, of course, the drug must be in either one or the other form. The total moles of drug in the just-clear liter of solution being 0.00625 as stated, the molar amount as drug-saccharate would be 0.00625 × 0.348 = 0.00217 moles. Postulating a 1:1 interaction, the molecular weight of the chlorpromazine saccharate = 502. Therefore, the grams of this complex per liter would be 502 × 0.00217 = 1.09 Gm./L.; this then should be the solubility of the complex. A solution containing both components at a concentration of 0.05 *M* each (17.7 Gm. drug hydrochloride plus 12.06 Gm. of sodium saccharin, *q.s. ad* 1 L.) was made so that the complex would be forced out of solution. The grams of complex possible would be 0.05 × 502 = 25.10 Gm.; 1.09 Gm. would be soluble as shown. Thus 24.01 Gm. should settle out; 24.0 Gm. was actually found.

Trifluoperazine and Saccharin.—Similarly to the above, the trifluoperazine dihydrochloride and saccharin case was treated probability-wise. This case differs somewhat from the chlorpromazine situation in that two moles of anion will surround the drug and the possibilities are altered. The drug may be surrounded by Cl-Cl, S-S, Cl-S, or S-Cl. Therefore, having found that the maximum permitted concentrations of the two components were 0.005 *M* drug and 0.00788 *M* saccharin (a drug:saccharin ratio of 1:1.576), the probabilities are: P_{Cl} = 1/2.576; Ps = 1.576/2.576. Hence the probabilities of the double combinations would be: $P_{Cl-Cl} = (1/2.576)^2 = 0.151$; $P_{S-S} = (1.576/2.576)^2 = 0.375$; $P_{Cl-S} = 2[1.576/(2.576)^2] = 0.474$. These probabilities again add up to 1.00, as the drug must be in the four mentioned forms; actually there are only three forms, ehnce the factor of 2 indicating that we could not distinguish between the Cl-S and S-Cl species. The total moles of drug in the just-clear liter of solution being 0.005, the molarity of drug-saccharate would be 0.005 × 0.375 = 0.001875 *M*. Postulating a 2:1 saccharin to drug interaction, the molecular weight of the trifluoperazine disaccharate = 774. Therefore, the grams of this complex per liter, *i.e.*, the solubility, would be 774 × 0.001875 = 1.45 Gm./L. A liter of solution containing 0.05 moles of trifluoperazine dihydrochloride (24.02 Gm.) and 0.10 moles of sodium saccharin (24.12 Gm.) was made. The grams of complex possible would be 0.05 × 774 = 38.7 Gm.; 1.45 Gm. would be soluble as shown. Thus, 37.25 Gm. should settle out; when the solution was made, 38.0 Gm. was actually found.

Before describing the work with prochlorperazine ethanedisulfonate and saccharin, which proved to be the most interesting case, some other comments are appropriate. All the complexes came out of solution as wet oils which when dried became hard glasses which could not be crystallized. [Incidentally, Japanese workers (17) were able to crystallize both mono- and di-saccharinates of thiamine. However, Hamilton and Turnbull (18) prepared many amine saccharinates, *i.e.*, substituted ammonium salts of saccharin, and found them generally difficult or impossible to crystallize.] An attempt to show that the complexes really existed in addition to their probability existence at concentrations lower than their maximum solubility was not successful. The method of continuous variations was employed, using the refractive indices of appropriate binary solutions as the characteristic property followed. The individual sodium saccharin and drug solutions *per se* exhibited easily measured changes in refractive index, but the binary solutions studied showed simple additive effects with no maxmin. This observation also supports the concept that the complexes in solution are somewhat elusive and consist essentially only of a certain ionic environment.

Prochlorperazine and Saccharin.—The probability analysis and subsequent precipitation study, in agreement in the two cases above, did not present a coherent picture in the prochlorperazine ethanedisulfonate–sodium saccharin case. It was evident

[1] Marketed as Thorazine, Compazine, and Stelazine, respectively, by Smith Kline & French Laboratories, Philadelphia, Pa.

that we were postulating the oil to be something that it was not. Therefore, it was decided to investigate in detail the composition of the oil.

The complex oil was made by mixing 0.3-*M* solutions of each component, separating the oil formed and drying it at 85°, whereupon it became a transparent, hard glass. The material was soluble in water and in alcohol with a strong acid reaction; it was insoluble in ether or chloroform. When a portion of the mass was dissolved in water and then neutralized with sodium hydroxide, a brown liquid which was acid- but not water-soluble separated. The physical appearance and solubility behavior paralleled those of prochlorperazine base. When two different aqueous complex solutions made from the glass were spectrophotometrically analyzed, the per cent of drug base in the oil was found to be 56.42% and 56.50%, for an average of 56.46%. Upon treating a small amount of the mass with dilute hydrochloric acid, a white, sweet precipitate was produced. The material melted in the range 227–230° and was obviously saccharin. Quantitative analysis for saccharin was done as follows: A sample of drug complex (1.519 Gm.) was dissolved in water, acidified (HCl), and extracted with chloroform-alcohol (9:1). The extract was evaporated to dryness, dissolved in water, neutralized with sodium hydroxide, and extracted with ether. The aqueous layer, containing the sodium saccharin and the sodium chloride, was evaporated, acidified, and extracted with chloroform-alcohol. The extract was evaporated to dryness and the residue taken up in water and titrated with 24.55 ml. of 0.0973 *N* sodium hydroxide. The per cent saccharin in the sample is then: $24.55 \times 0.0973 \times 183.2 \times 100/1519 = 28.82\%$.

Thus, it appeared that there was about 15% of a third component in the drug-saccharin complex. Evidence was soon obtained which indicated that there was another acid in the product: When the complex in water was titrated with base in the presence of ether (to remove the organic base), the amount of alkali needed was much more than that required for neutralization of the 29% saccharin. Naturally, the conclusion was that ethanedisulfonic acid was also present in the oil; this was verified, as will be shown.

The various possible drug-acid-saccharin interaction products were now considered. The one that fit the data was a complex consisting of 2 moles of saccharin, 2 moles of prochlorperazine, and 1 mole of ethanedisulfonic acid; this would contain about 57% drug, 28% saccharin, and 15% ethanedisulfonic acid. A titration made as described in the last paragraph indicated, after adjusting for the theoretical amount of saccharin in the postulated complex, that the ethanedisulfonic acid comprised about 12.5% of the complex. A further check on the acid content was made by mixing 5.64 Gm. (0.01 mole) of prochlorperazine ethanedisulfonate with 2.41 Gm. (0.01 mole) of saccharin sodium. After complex formation, the aqueous solution should contain 1.17 Gm. of disodium ethanedisulfonate; experimentally we recovered 1.02 Gm. of a white powder which was soluble in water, insoluble in alcohol, neutral to litmus paper as an aqueous solution, and had an equivalent weight of 116.5 (theoretical = 117.1). The equivalent weight was determined by running the sample alongside a known sample of disodium ethanedisulfonate through an analytical grade of cation exchange resin[2] and titrating the eluate with alkali. The known gave an equivalent weight of 116.8, the unknown, 116.5. The presence of the acid in solution corroborated the chosen composition of the complex, as it was consistent with the rest of the data, and it eliminated some alternate possibilities. Thus it appears that one molecule of ethanedisulfonic acid connects two prochlorperazine-saccharin interaction products.

As mentioned, the product defied all attempts to crystallize it. However, it was possible to obtain a powder (amorphous) by freeze-drying a solution. The material thus obtained was then checked for carbon-hydrogen and, for a compound which would not crystallize and must be obtained as a wet oil initially, the agreement is quite satisfactory.

Anal.—Calcd. for $C_{56}H_{64}Cl_2N_8O_{12}S_6$: C, 51.56; H, 4.95. Found: C, 53.44, 53.65; H, 4.95, 5.26.

CONCLUSIONS

The nature of the ionic atmosphere around a drug in solution may explain why different salts of the same drug can exhibit different stability characteristics.

The composition of the ionic atmosphere may be determined by examining the concentrations of all the species involved and using simple probability mathematics; the species involved may be changed and thus studied by varying the salt types in the solution.

Although the ionic atmosphere forms what may be termed a "probability complex" detectable only mathematically, these probability complexes can be forced out of solution and their composition verified.

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[2] Analytical Grade Amberlite IR-120 (H), marketed by Rohm & Haas Co.

Substituted Salicylanilides II

Further Antimicrobial and Other Studies with Halonitrosalicylanilides

By ROBERT G. TABORSKY† and ROLAND J. STARKEY

A detailed examination of the general antimicrobial spectra of halonitrosalicylanilides previously reported upon is described together with some general observations. Some other biological data are also presented.

THE OBSERVATION of antimicrobial activity among certain halonitrosalicylanilides has been previously reported (1). These compounds can be represented by the general formula shown in Fig. 1.

Since that report, a more detailed examination of the general spectra of antimicrobial action of the series has been made. In addition, data have also been obtained upon some areas of the general biological activity of these compounds together with further toxicity data. These results are reported below, together with some general observations.

It was of interest to determine the potencies of one of the more active members of each of the 3-nitro and the 5-nitro series of compounds against a variety of fungi and bacteria. 4′-Chloro-3-nitrosalicylanilide (I) from the former series and 4′-bromo-5-nitrosalicylanilide (II) from the latter series were chosen (see Fig. 2).

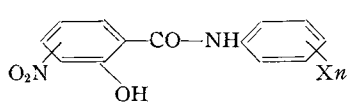
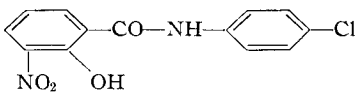
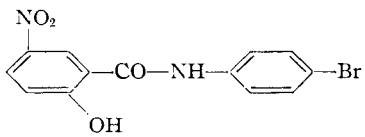


Fig. 1.—General formula for halonitrosalicylanides where X is Br, Cl, F or I and n = 1, 2, or 3.



(I)



(II)

Fig. 2.—Structural formulas for the two compounds studied.

These two compounds were evaluated against a number of interesting microorganisms by determining the minimum concentrations required to cause inhibition of their growth. The data are presented in Table I.

On the basis of these data, it can be seen as had already been observed in the preliminary study that with only a few exceptions, the 5-nitro compound is a more potent antimicrobial than the 3-nitro compound.

In vivo, activity of 4′-chloro-5-nitrosalicylanilide against *Mycobacterium leprae* was determined in mice. The results of the study with a streptomycin control are presented in Table II.

A degree of activity was clearly evident. However, the activity was of lesser magnitude than the control antimicrobial.

In a further study with two related organisms, *Mycobacterium tuberculosis* and *Mycobacterium avium*, and comparison with *Salcide*, employing the serial broth dilution method, threshold values of 5 mcg./ml. were obtained. *Salcide* is a commercial preparation containing approximately equal amounts of 3′-chloro-3-nitrosalicylanilide, 3′-chloro-5-nitrosalicylanilide, 4′-chloro-3-nitrosalicylanilide, and 4′-chloro-5-nitrosalicylanilide.

Thirteen of the halonitrosalicylanilides were then compared in activity against two Gram-positive and one Gram-negative bacteria and a fungus. A mixture of two of the anilides was also examined. These results are shown in Table III. Several interesting facts become apparent upon studying the data. In the first place, as had been initially observed to a limited degree, the compounds were more potent against the Gram-positive organisms than the particular strain of *E. coli* used.

Manifestation of synergism is seen upon comparison of the value against *Aspergillus niger* for the mixture with the values for the unmixed materials. Similar synergisms were encountered with other mixtures and are referred to below. Comparison of the values for the 3-nitro and 5-nitro compounds unequivocally demonstrates the consistently higher potencies of the latter. Finally, it was possible to select on the basis of these results, the most active and interesting members of the series for further study. These were 3′-chloro-5-nitrosalicylanilide, 4′-chloro-5-nitrosalicylanilide, and 4′-iodo-5-nitrosalicylanilide.

To further our knowledge about the halonitrosalicylanilides and to learn more about the synergism between some of the members, several experiments were designed involving mixtures of the compounds. Because of the many combinations possible with all 13 of the active halonitrosalicylanilides, only those exhibiting the highest degree of activity were selected

Received March 5, 1962, from the Ben Venue Laboratories, Inc., Bedford, Ohio.

Accepted for publication June 6, 1962.

We wish to extend our gratitude to Dr. John Erlich of the Parke Davis Company, who carried out some of the antimicrobial determinations and to Dr. Y. T. Chang of the Public Health Service, who screened several of the halonitrosalicylanilides against *Mycobacterium leprae* in mice. Our appreciation is also extended to the National Cancer Screening Service for their screening of the halonitrosalicylanilides for antitumor activity.

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TABLE I.—QUANTITATIVE ANTIMICROBIAL DETERMINATIONS FOR TWO HALONITROSALICYLANILIDES BY SERIAL BROTH DILUTION

	Antifungal *in Vitro* Test Results[a] (Broth Dilution)					
	4′-Chloro-3-Nitrosalicylanilide			4′-Bromo-5-Nitrosalicylanilide		
	Inhibition			Inhibition		
	Complete	Partial	None	Complete	Partial	None
B. dermatitidis	>200	200	100	6.25	3.13	>1.56
C. albicans	100	...	50	200	...	100
C. neoformans	25	12.5	6.25	3.13	...	>1.56
H. capsulatum (yeast phase)	100	50	25	6.25	...	3.13
N. asteroides	3.13	...	1.56	1.56	...	>1.56
T. interdigitale	6.25	3.13	1.56	3.13	1.56	>1.56

	Antibacterial *in Vitro* Test Results[a] (Broth Dilution)	
	Turbidimetric, 50% Inhibition	Turbidimetric, 50% Inhibition
A. aerogenes	>20	15
A. tumefaciens	0.83	1.1
B. firmus	0.71	1.1
E. coli (2 strains)	12. ->20	3.8
K. pneumoniae (3 strains)	8. ->20	3.3
P. vulgaris (2 strains)	14. ->20	7.3
S. schottmuelleri	16	14
S. typhimurium	19	10
S. typhosa	>20	13
S. paradysenteriae	10	5
S. sonnei	>20	16
S. aureus (4 strains)	0.35–3.7	0.68–1.1
S. infrequens	9.7	2.1
V. comma	1.8	1.5

[a] All of the results are expressed as the concentration of antimicrobial in the microbial growth broth in mcg./ml. which produces inhibition.

TABLE II.

Treatment of the Animals	Dose	Leprosy Score in Mesentery[a]
Leprosy control untreated	...	4.7
Streptomycin	2 mg. subcutaneously	2.7
4′-Chloro-5-nitrosalicylanilide	0.1% in the diet	3.5

[a] The score is based upon a count of the number of infected cells in the mesentery (2).

for the synergistic studies. Quite by accident, a mixture exhibiting a very high degree of synergistic action was initially prepared consisting of 3 parts of 3′-chloro-5-nitrosalicylanilide and 1 part of 4′-iodo-5-nitrosalicylanilide. In an initial experiment, the threshold inhibition values were determined for each of the two unmixed materials and for the three-to-one mixture against six different microorganisms. The results of this study are summarized in Table IV. It can be seen that the synergism appears to be exhibited against fungi, but not against bacteria. A particularly resistant strain of *E. coli* had been used in these studies for the sake of detecting any synergistic action.

Other mixtures were examined in order to determine whether any generalizations about the types of structural combinations exhibiting synergism might be possible. The three-to-one mixture was adhered to rather arbitrarily since initially a high degree of synergism had been observed with that ratio. *Aspergillus niger* was used throughout as the test organism since it had exhibited the greatest response to the initial synergistic ratio tried in preliminary studies. The results are presented in Table V.

TABLE III.—*In Vitro* ANTIMICROBIAL ACTIVITY OF A GROUP OF SUBSTITUTED HALONITROSALICYLANILIDES[a]

Compound	*Staphylococcus aureus* (MGH #1)[b]	*Escherichia coli* (ATCC10740)	*Bacillus subtilis* var. *globigii*	*Aspergillus niger*[c]
3′-Chloro-5-nitrosalicylanilide	5.2–10	>300	30–40	>100
2′-Chloro-5-nitrosalicylanilide	20–25	>300	10–15	>100
4′-Iodo-5-nitrosalicylanilide	3.4–5.2	>300	>10	...
4′-Bromo-5-nitrosalicylanilide	5–10	...	10–15	...
4′-Fluoro-5-nitrosalicylanilide	>10	200–300	5.2–10	>100
2′,4′-Dichloro-3-nitrosalicylanilide	40–50	>300	>70	>100
4′-Fluoro-3-nitrosalicylanilide	>60	...	...	...
2′-Chloro-3-nitrosalicylanilide	5.2–10	...	...	>100
3′-Chloro-3-nitrosalicylanilide	>60	>300	>70	>100
4′-Chloro-3-nitrosalicylanilide	20–25	...	>70	...
4′-Bromo-3-nitrosalicylanilide	20–25	>300	15–20	>100
4′-Chloro-5-nitrosalicylanilide	5.2–10	...	30–40	>100
4′-Chloro-5-nitrosalicylanilide and 4′-Chloro-3-nitrosalicylanilide (3:1)	>10	>300	50–60	50–60
2′,4′-Dichloro-5-nitrosalicylanilide	>10	>300	10–15	>100

[a] Tube dilutions are plated on nutrient agar following 72 hours' incubation at 37° and antimicrobial activity is expressed as the lowest concentration (mcg./ml.) which completely inhibits the growth of a specific test organism. [b] Penicillin resistant strain of *Staphylococcus aureus* obtained from Metropolitan General Hospital, Cleveland, Ohio. [c] Plate-agar dilution assay.

TABLE IV.—THE ANTIMICROBIAL ACTIVITY OF UNMIXED AND MIXED 3′-CHLORO-5-NITROSALICYLANILIDE AND 4′-IODO-5-NITROSALICYLANILIDE[a]

Organism	Activity, mcg./ml. Unmixed 3′-Chloro-5-nitrosalicylanilide	Unmixed 4′-Iodo-5-nitrosalicylanilide	3:1 Mixture of the Chloro and Iodo Compounds
Streptococcus pyogenes (C203)	>2-<10	>10-<20	>10-<20
Streptococcus pyogenes	10–15	5–10	10–15
Escherichia coli	>600	>700	>600-<700
Aspergillus niger	>600	400-500	15–20
Memnoniella echinata	>600	>600	10–20
Aspergillus terreus	>600	>600	600

[a] Minimum-plate dilution for fungi and minimum-tube dilution for bacteria.

TABLE V.—ACTIVITIES OF VARIOUS 3:1 MIXTURES OF HALONITROSALICYLANILIDES *vs. Aspergillus niger*[a]

Mixtures	Parts	Minimum Threshold Concentration for Inhibition mcg./ml.
2′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-3-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	400
4′-Fluoro-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	20–30
4′-Chloro-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	15–20
4′-Iodo-5-nitrosalicylanilide	1	
4′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-5-nitrosalicylanilide	1	

[a] In all cases, the unmixed components had values of over 600 mcg./ml.

The most interesting conclusion obtained from Table V is the great specificity of the various components of the mixtures for synergism. In this limited current study of synergism, it can be generalized that only mixtures of halo-5-nitrosalicylanilides where one component has a halogen in the 3′-position and the other in the 4′-position exhibit any appreciable degree of synergistic activity.

Our investigations with the halonitrosalicylanilides then took another direction in an attempt to discover some more general biological characteristics of the materials. It was of interest to determine the fate of the material when introduced into an animal by the oral route. The rabbit was selected as a suitable animal and suspensions of 3′-chloro-5-nitrosalicylanilide in gum acacia were administered orally. In these studies, gross symptoms of toxicity such as emesis, diarrhea, or weight loss were not detected at daily dosages of 1 Gm. per Kg. for a 1-week period.

Pooled feces of the rabbits were extracted with dimethylformamide, a solvent in which 3′-chloro-5-nitrosalicylanilide is extremely soluble. The extractions were filtered and evaporated to dryness and tested for antimicrobial activity *vs. Staphylococcus aureus*, one of the organisms very sensitive to this series. No trace of antimicrobial activity was detected for the residue. Thus the conclusion is reached that either the substituted salicylanilide is chemically modified in the gastrointestinal tract or else is very slowly absorbed into the system at sublethal levels.

In our original publication and in subsequent studies, we had found that generally the LD_{50} in the rat, for the halo-3-nitrosalicylanilides by the intraperitoneal route was in the order of 35 mg. per Kg. and that this value was 125 mg. per Kg. for the halo-5-nitrosalicylanilides. The oral feeding study mentioned above was carried out upon only one animal. Therefore, it cannot by any means, be considered a toxicity study. However, it does serve as an indication that the oral doses which are tolerated are many times greater than the intraperitoneal dose.

Because antimicrobial materials, particularly antibiotic broths, have often exhibited antitumor activity, all of the halonitrosalicylanilides prepared in our studies were submitted to the National Cancer Screening Service for a primary antitumor screening against four tumor systems in mice.

Some of the members exhibited varying degrees of antitumor activity in these tests. 4′-Iodo-5-nitrosalicylanilide qualified for further examinations but its activity was not considered consistent enough for more extensive studies. The results of that study are shown in Table VI.

TABLE VI.—REDUCTION OF TUMOR WEIGHT BY 4′-IODO-5-NITROSALICYLANILIDE[a]

Experiment	Daily Dose, mg./Kg.	Survivors	Tumor Weight—Medicated Animals	Tumor Weight—Non-medicated Animals	Reduction, %
I	113	6/6	5.5	8.2	67
II	113	6/6	1.2	7.7	15
III	113	4/6	8.2	7.8	105
IV	113	5/6	1.5	6.4	23
V	113	5/6	4.3	8.6	50
VI	113	3/6	4.5	8.8	51
VII	113	8/10	7.7	7.6	101

[a] The Erlich Ascites Tumor was used throughout.

DISCUSSION AND SUMMARY

The spectra of certain halonitrosalicylanilides have been investigated to show that they possess bactericidal and fungicidal activity against a large variety of microorganisms.

Upon comparing the members among themselves against five organisms, 3′-chloro-5-nitrosalicylanilide (I), 4′-chloro-5-nitrosalicylanilide (II), and 4′-iodo-5-nitrosalicylanilide (III) emerged as the three most active compounds. It was further of interest, to discover that mixtures of I and III and of II and III had activities as great as 30 times that of the unmixed materials against *Aspergillus niger* and *Memnoniella echinata*. The synergism was observed against fungi in general, but not bacteria. It was,

however, exhibited by several other mixtures to some degree, but only the above-mentioned combinations had a very high degree of activity in this respect.

In further studies upon general biological activity, it was found that the oral toxicity of 3′-chloro-5-nitrosalicylanilide may be many times less than by intraperitoneal administration.

An attempt to correlate pKa and antimicrobial activity for several different salicylanilides has been made (3). The hypothesis put forth was that pKa would be a measure of chelating ability and that, in effect, a correlation of pKa and activity was a correlation of chelating power and activity. In our own studies it was demonstrated that indeed the halonitrosalicylanilides were powerful chelating agents. Upon taking a solution of 5-nitrosalicylic acid and adding ferric chloride solution, a brilliant blood-red chelate complex is formed. However, when EDTA is added to the solution, the color is immediately discharged indicating the transferral of Fe^{+++} to form an EDTA-Fe^{+++} relatively colorless complex. However, when this was repeated with 4′-chloro-5-nitrosalicylanilide, the red color that formed could not be discharged by means of EDTA indicating the former to be a better chelater of iron than EDTA. It must be remembered, on the other hand, that excellent chelating agents such as EDTA do not exhibit general antimicrobial activity and that chelation alone does not give rise to antimicrobial activity. Furthermore, among some of the original halonitrosalicylanilides prepared by ourselves were a series of halo-3,5-dinitrosalicylanilides whose phenolic hydrogens would have a higher pKa than those of the mono-nitrated products. However, these compounds were completely devoid of activity against bacteria or fungi. Thus it is suggested that the requirements for a substituted salicylanilide to possess activity could logically involve its chemical functionality, general solubility, and size and shape of the molecule. These factors could affect its migration across physiological barriers and its ability to seat in enzyme sites which in addition to chelating ability, could determine or influence antimicrobial activity.

Currently, new substituted salicylanilides are being prepared with the idea of exploring some of the molecular requirements for activity. In these studies, particular emphasis is being placed upon investigating the relationship between chelating ability and activity together with some of the other considerations mentioned above. Also, because of the very specific type of synergism observed, a further investigation is underway in attempting to further determine its limitations and to theorize upon its origin. It is hoped that from these investigations more information may be obtained regarding the mechanistic and theoretical aspects of the antimicrobial action of variously substituted salicylanilides.

EXPERIMENTAL

Quantitative *in Vitro* Antibacterial Assays.—These were conducted turbidimetrically by the serial broth dilution method. Inhibition of growth was reported as complete, 50%, and incomplete and measured as the mcg./ml. concentration to cause the inhibition. In some instances absolute bactericidal end points were determined visually but confirmed by streaking replicate sections of nutrient agar plates with several dilutions from the test series.

Stock solutions of halonitrosalicylanilides were prepared by dissolving 100 mg. in 100 ml. of distilled water and adjusted to pH 7.3 with 0.1 *N* sodium hydroxide with some compounds requiring heating to 50° to obtain complete solubilization. The solutions were then sterilized in serum vials at 15 pounds pressure for 15 minutes.

One milliliter aliquots were removed with tuberculin syringes equipped with #22 gauge needles and aseptically transferred to vials containing 9.0 ml. of fluid thioglycollate (BBL). Subsequent dilutions were made in a similar manner. Each test series included viability and media controls.

The inoculum was prepared by making a minimum of three transfers of the test organism in fluid thioglycollate and inoculating 0.05 ml. of the test organism into each vial following 18 hours incubation at 37°. Readings were made at the conclusion of 24 and 72 hours of incubation.

Quantitative *in Vitro* Antifungal Assays.—These were conducted by the broth dilution turbidimetric and agar-dilution linear-growth methods. The former assay was performed in a manner similar to that described for determining antibacterial activity, but with the exception of using fluid dextrose Sabouraud throughout.

In the linear-growth technique, dilutions of the appropriate stock solution (0.001 Gm./ml.) were added to sterile plastic Petri dishes (100 x 15 mm.), and the final volume adjusted to 10 ml. with Sabouraud dextrose agar.

Following solidification, one drop of a concentrated spore suspension was placed in the center of each plate with a 1.0-ml. serological pipet. Controls consisted of untreated Sabouraud agar, though inoculated with the test fungus.

Diameter of the fungus on untreated plates was compared for 7 days with those treated with varying concentrations of a halonitrosalicylanilide. All plates were incubated at room temperature with complete antifungal activity expressed in micrograms per milliter.

Oral Fate of 3′-Chloro-5-nitrosalicylanilide.—A 2.9 Gm. quantity of 3′-chloro-5-nitrosalicylanilide was suspended in 40 ml. of a 10% suspension of gum acacia. This was slowly administered to a 2.9 Kg. rabbit by gastric intubation and was repeated on 5 consecutive days. Side effects or other indications of acute toxicity were not observed.

All of the feces collected during this period, approximately 200 Gm. were extracted by a total of 1.0 L. of dimethylformamide. Portions were combined, vacuum filtered, and evaporated to dryness in a small glass still at 60° and 5 mm. A 30% concentration of the residue was prepared in distilled water and screened against *Staphylococcus aureus* by the cup-plate method. No antimicrobial activity was observed. Controls of 3′-chloro-5-nitrosalicylanilide (0.001 Gm./ml.) elicited conspicuous zones of inhibition.

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False-Positive Alkaloid Reactions Obtained with Extracts of *Piper methysticum*

By A. R. FURGIUELE, N. R. FARNSWORTH, and J. P. BUCKLEY

Piper methysticum Forst. (Piperaceae) has been reported by several investigators to contain alkaloids. Several screening methods utilized in this study substantiated these findings; however, after comparing a variety of test procedures, these "positive" alkaloid tests were shown to be due to certain non-nitrogenous α-pyrone compounds. The α-pyrones were also demonstrated to react with a modified Dragendorff spray reagent, after thin-layer chromatography on silica gel G plates. The orange color produced after spraying these α-pyrones was typical of the usual alkaloid positive result, but the color persisted for less than 24 hours. The use of heat to dissolve extracted residues in 1% hydrochloric acid for testing was shown to intensify the alkaloid-like reactions. The results are discussed relative to the screening methods employed.

A NUMBER of reference sources have indicated that the common alkaloid precipitating reagents (Mayer's, Wagner's, Dragendorff's, Sonnenschein's, Hager's, *etc.*) are capable of precipitating certain nonalkaloid materials from solution. Certain proteins, amino acids, glycosides, tannins, betaines, choline, carbohydrates, and ammonium salts have most frequently been implicated as substances present in plants that will give such false-positive reactions (1–11).

Piper methysticum Forst. is a plant indigenous to many islands of the South Pacific. The roots of this plant have been used in the form of a beverage, known as Kava, Kawa, or Awa, by natives of many of these islands to allay anxiety and reduce fatigue (12). Other uses claimed for Kava have been as an aphrodisiac, antiseptic, tonic, anodyne, sudorific, diuretic, stimulant, and intoxicant (13). Local application of the drug is reported to produce a burning pain followed by loss of sensitivity (14). Perhaps the most significant biological effects produced by extracts of this plant are the induction of intoxication of a silent and drowsy nature, accompanied by incoherent dreams when large doses are ingested (13).

Six α-pyrone compounds have been isolated from Kava. They are kawain (*K*), dihydrokawain (*DHK*), methysticin (*M*), dihydromethysticin (*DHM*), yangonin (*Y*), and desmethoxyyangonin (*DMY*), (15–20). See Fig. 1. Methysticin and dihydromethysticin have been demonstrated to be the most effective sedative compounds of the six pyrones isolated to date (20). Recently, Hansel, *et al.* (21), have isolated two new compounds, flavokawin *A* and flavokawin *B* from Kava, but these were shown to be devoid of sedative properties. In addition, gum, starch, sugar, resins, glucosides (22), and alkaloids (22–24) have been reported to be present in Kava.

Since the alkaloids reported present in this plant had not been thoroughly investigated, our initial studies were directed to the isolation, separation, and identification of these compounds. The present report is concerned with evidence pointing to a class of non-nitrogenous chemical substances present in *P. methysticum* that render alkaloid-positive reactions with a number of common alkaloid-detecting reagents.

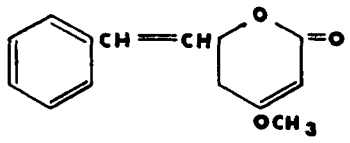
EXPERIMENTAL

Materials.—The plant material used in this investigation was collected in Hawaii during the fall of 1959 and consisted of the roots of *Piper methysticum* Forst. (Piperaceae).[1] The *Hamamelis virginiana* L. and *Prunus serotina* Ehrh. used as alkaloid negative controls were obtained commercially as powdered leaves and bark, respectively.[1] Thin-layer chromatography experiments were conducted using the DeSaga apparatus,[2] designed to prepare a 200 μ matrix on 200 x 200 mm. glass plates. The adsorbent matrix was prepared from silica gel G[2] with all prepared plates activated at 105° for 30 minutes, followed by cooling to room temperature in a soda-lime desiccator. All solvents and chemicals used in this investigation were of reagent grade quality unless otherwise specified.

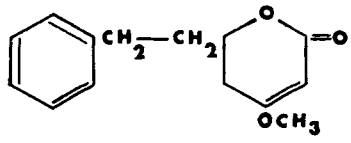
Comparison of Screening Methods.—All attempts made to isolate crude alkaloids from Kava by the usual methods employed in phytochemical studies were unsuccessful. Since the possibility existed that alkaloids were not present, small samples were screened for alkaloids according to the methods of Wall, *et al.* (25, 26), Wall method; Kiang and Douglas (27), Kiang method; and Swanholm, *et al.* (24), Swanholm method. Using the reagents to detect alkaloids that were recommended for each method, it was found that pre-

Received March 29, 1962, from the Departments of Pharmacology and Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh 13, Pa.
Accepted for publication June 14, 1962.
This investigation was supported by grant MY-3029, NIMH, Bethesda, Md.

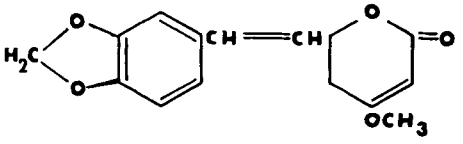
[1] Supplied through the S. B. Penick and Company, New York 8, N. Y.
[2] Distributed by Brinkmann Instruments Inc., Great Neck, L. I., N. Y.



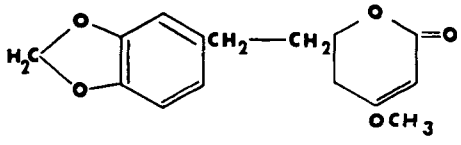
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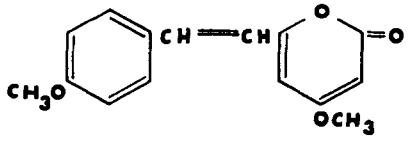
DHK



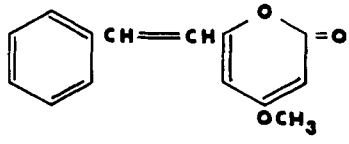
M



DHM



Y



DMY

Fig. 1.—Structures of *P. methysticum* α-pyrones: kawain, (*K*); dihydrokawain, (*DHK*); methysticin, (*M*) dihydromethysticin, (*DHM*); yangonin, (*Y*); desmethoxyyangonin, (*DMY*).

liminary tests for the alkaloids using the Wall method were positive, while confirmatory tests were negative. The Kiang method indicated that alkaloids were present corresponding to *ca.* 0.01% (as brucine hydrochloride). This is in general agreement with the reported occurrence of alkaloids in Kava of 0.022% by Winzheimer (22) and 0.012% by Scheuer and Horigan (23). The methods utilized for alkaloid estimation were not indicated by these investigators. Both Swanholm methods (direct hydrochloric acid extraction and Prollius extraction) gave results indicative of the presence of alkaloids in this plant.

Since the confirmative test by the Wall method was negative, and the methods of Kiang and Swanholm were indicative of the presence of alkaloids, a series of experiments were designed to clarify these conflicting results. Preliminary experiments had led us to believe that the material possibly responsible for the positive tests was chloroform-soluble. Therefore, it was decided to simultaneously compare the results obtained by screening *P. methysticum* and two plants known to be devoid of alkaloids, or alkaloid-like material, but to which chloroform extracts of *P. methysticum* had been added.

The Swanholm method was utilized and the leaves of *H. virginiana* and the bark of *P. serotina* were included as controls. It was necessary to include controls in this study since extraneous plant materials (resins, pigments, *etc.*) present in the extracts could alter the separation and/or resolution of the α-pyrones present in the *P. methysticum* chloroform extractives. Four 5-Gm. samples of finely powdered *P. methysticum* roots were continuously extracted with chloroform for 3 hours in suitable Soxhlet

TABLE I.—ALKALOID SCREENING RESULTS OF *P. methysticum* AND *P. methysticum* CHLOROFORM-SOLUBLE TREATED CONTROLS

Reagent	*P. methysticum* Method A[a]		*H. virginiana* + *P. methysticum* Method A[a]	B[b]	*H. virginiana* Method A[a]	B[b]	*P. serotina* + *P. methysticum* Method A[a]	B[a]	*P. serotina* Method A[a]	B[b]
Mayer's	tr	tr	tr	tr	—	—	tr	tr	—	—
Wagner's	+	+	+	+	—	—	+	+	—	—
Picric acid	tr	tr	tr	tr	—	—	tr	tr	—	—
Dragendorff's	+	+	+	+	—	—	+	+	—	—
Sonnenschein's	++	++	++	++	—	—	++	++	—	—
Silicotungstic acid	tr	tr	tr	tr	—	—	tr	tr	—	—

[a] A, acid extract; [b] B, Prollius extract. Key: ++, moderate precipitate; +, light precipitate; tr; trace (turbidity); —, no turbidity or precipitate.

extractors. Two of these chloroform extracts were mixed with separate 5-Gm. samples of finely powdered *H. virginiana* leaves and two were mixed with separate 5-Gm. samples of powdered *P. serotina* bark. These four samples were then heated to dryness on a steam bath.

One set of samples consisting of 5 Gm. each of *P. methysticum*, *H. virginiana* treated with *P. methysticum*, *H. virginiana*, *P. serotina* treated with *P. methysticum*, and *P. serotina* was screened by the Swanholm method utilizing direct 1% hydrochloric acid extraction. The samples were mixed with sufficient 1% hydrochloric acid to form a slurry, macerated at 80° for 4–6 hours, cooled to room temperature and filtered. Each filtrate was adjusted to 2 ml. by passing 1% hydrochloric acid through the marc on the filter paper.

A duplicate group of 5-Gm. samples was extracted with *ca.* 30 ml. of Prollius fluid (ether/chloroform/ethanol/ammonia) (25:8:2.8:1)(v/v) for 56 hours at room temperature in tightly stoppered flasks. After the maceration period, each sample was filtered and the residue on each filter paper was washed with an additional 20 ml. of Prollius fluid. The filtrates were then evaporated to dryness on a steam bath. Each residue was then mixed with *ca.* 4 ml. of 1% hydrochloric acid and allowed to macerate at 80° for 1 hour with occasional stirring. After cooling to room temperature, the extracts were filtered and sufficient 1% hydrochloric acid added to each sample to make 2 ml.

The samples were each tested for alkaloids by the addition of 0.1 ml. of reagent to 0.2 ml. of extract (equivalent to 0.5 Gm. of dried plant material). Mayer's, Wagner's, picric acid, Dragendorff's, Sonnenschein's, and silicotungstic acid reagents were used. The results are presented in Table I and clearly indicate the presence of material in the chloroform-soluble *P. methysticum* extracts that produce precipitates with the reagents indicated.

Since most alkaloids are known to exist in plants as chloroform-insoluble salts, the presence of chloroform-soluble, nonalkaloid material as being responsible for the alkaloid-like reactions was further suspected.

For comparative purposes, duplicate samples of *P. methysticum* were screened by the Prollius extraction method with one sample treated as previously described, using heat to aid in dissolving the Prollius residue in 1% hydrochloric acid. The second sample was treated in a similar manner; however, no heat was applied. This evaluation was considered necessary since the original Webb method (11) was found to give positive alkaloid tests with *P. methysticum* by direct acid extraction (heat applied at 60° for 1/2 to 1 hour), but negative tests by the Prollius method in which heat was not applied. When *P. methysticum* was screened by the Arthur modification (28) of the Webb method, negative results were obtained with the direct acid extraction (no heat) as well as with the Prollius extraction (no heat). The results of this study are indicated in Table II and point out the effect of heat on increasing the false-positive alkaloid reactions with these reagents.

TABLE II.—THE EFFECT OF HEAT ON *P. methysticum* PROLLIUS EXTRACTION ALKALOID SCREENING RESULTS

Reagent	*Piper methysticum*[a] Heat	No Heat
Mayer's	tr	—
Wagner's	+	—
Picric acid	tr	—
Dragendorff's	+	—
Sonnenschein's	++	+
Silicotungstic acid	tr	—

[a] ++, moderate precipitate; +, light precipitate; tr, trace (turbidity); —, no turbidity or precipitate.

Thin-Layer Chromatography Studies.—It is common practice in many laboratories to detect alkaloids in plant material by means of chromatographic methods using a modified Dragendorff spray reagent. After chromatograms are treated with the Dragendorff spray, the appearance of typical yellow-orange to orange spots is generally accepted as good evidence of the presence of alkaloids in the material chromatographed. Since the standard Dragendorff reagent produced a visible alkaloid-like reaction with extracts of *P. methysticum* in test tube experiments, it was decided to determine the effect of the Munier and Macheboeuf modified Dragendorff spray reagent (29) on chromatograms prepared from the previously described Prollius extracts, in addition to certain controls.

An additional aid in allowing us to verify that chloroform-soluble *P. methysticum* extracts contained material responsible for false-positive alkaloid reactions was found in treating chromatograms of these extracts with sulfuric acid. Chloroform extracts of *P. methysticum*, in addition to the known α-pyrones isolated from this plant (chloroform-soluble), were found to react with sulfuric acid to produce red or yellow colors.

Silica gel G plates were spotted with separate solutions containing 1γ/1λ of each of the known α-pyrones (kawain, dihydrokawain, methysticin, dihydromethysticin, desmethoxyyangonin, yangonin) in chloroform. In addition, a solution containing a mixture of 1γ/1λ of each was prepared. Ten lambda of each solution was applied and a mixture of Skelly B/ethyl acetate (1:1) was used as the eluent. After proper development, the plates were removed from the chambers, air-dried and sprayed with sulfuric acid. The areas of color formation were quickly traced and the colors noted. All compounds reacting with sulfuric acid to produce a yellow color quickly faded, while those components producing red colors were more permanent. The result of this chromatographic separation is illustrated in Fig. 2. A suitable solvent system for the complete resolution of the six α-pyrones could not be devised, but in mixtures of appropriate concentration, differences in migration, combined with the sulfuric acid color reaction, were sufficient to allow detection of desmethoxyyangonin (yellow) and yangonin (yellow) from mixtures of kawain (yellow)-dihydrokawain (yellow) and methysticin (red)-dihydromethysticin (red).

Several dilutions of the known α-pyrones were prepared, applied to duplicate silica gel G plates, and chromatographed as previously described. One set of plates was sprayed with sulfuric acid and the other set with the Munier and Macheboeuf-Dragendorff spray in order to ascertain the minimal quantities detectable with each reagent. The results are indicated in Table III.

The initial reaction of the α-pyrones to the Dragendorff spray was quite similar to that produced by known alkaloids; however, it should be pointed out that the reaction was not of long duration. The initial orange color faded to yellow within 1 hour and, dependent on concentration, was usually rendered colorless after standing for 24 hours. This is in sharp contrast to most alkaloids that we have treated in a similar manner, in which the orange color has been observed to persist for several weeks.

Duplicate silica gel G plates were prepared for thin-layer chromatography, by the application of 100λ each of the 1% hydrochloric acid solutions of the Prollius residues, prepared as previously described. These solutions included *P. methysticum*, *H. virginiana* treated with *P. methysticum*, *H. virginiana*, *P. serotina* treated with *P. methysticum*, *P. serotina*, *P. methysticum* chloroform-soluble extract in 1% hydrochloric acid (unheated), and *P. methysticum* chloroform-soluble extract in 1% hydrochloric acid (heated). In addition, 20λ of a chloroform extract of *P. methysticum* containing the equivalent of 1 Gm. of drug in 50 ml., and 20λ of the α-pyrone mixture (1γ/1λ) in chloroform were applied. Both plates were eluted with Skelly B/ethyl acetate (1:1) as previously described and were air-dried. One plate was sprayed with sulfuric acid and the duplicate plate with the Munier and Macheboeuf-Dragendorff spray. The results are illustrated in Figs. 3 and 4 and point out that the known α-pyrones, present in chloroform extracts of *P. methysticum*, react with Dragendorff's spray reagent. Sufficient quantities of these compounds are soluble in heated 1% hydrochloric acid, as indicated by the chromatographic results, to account for the previously described alkaloid-like reactions

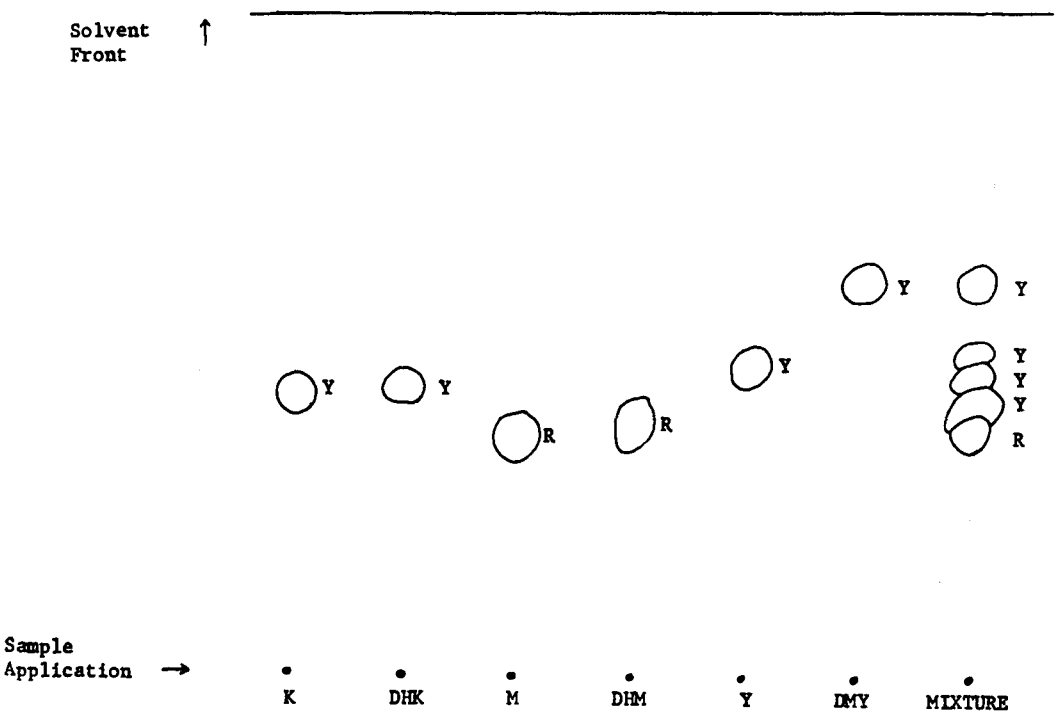


Fig. 2.—Thin-layer chromatogram of the six known *P. methysticum* α-pyrones in chloroform. Key (after sulfuric acid spray): Y = yellow, R = red.

TABLE III.—THE SENSITIVITY OF THE KNOWN *P. methysticum* α-PYRONES TO SULFURIC ACID AND DRAGENDORFF SPRAY REAGENTS[a]

α-Pyrone	Sulfuric Acid Spray: Minimal Detectable Quantity (γ) *ca.*	Sulfuric Acid Spray: Color	Dragendorff Spray: Minimal Detectable Quantity (γ) *ca.*	Dragendorff Spray: Color
Kawain	1	Yellow	10	Orange, fades to yellow
Dihydrokawain	4	Yellow	15	Orange, fades to yellow
Methysticin	1	Pink to red	10	Orange, fades to yellow
Dihydromethysticin	1	Pink to red	10	Orange, fades to yellow
Yangonin	1	Yellow	10	Orange, fades to yellow
Desmethoxyyangonin	4	Yellow	15	Orange, fades to yellow

[a] After thin-layer chromatography on silica gel G plates.

using Prollius extracts prepared with heat. Extracts prepared without the use of heat were only slightly soluble in 1% hydrochloric acid as evidenced by these results and undoubtedly the concentrations present in these Prollius extracts were too low to be detected by the precipitating agents employed in this study.

DISCUSSION

The results of this investigation clearly indicate that certain α-pyrone derivatives in *P. methysticum* can induce false-positive alkaloid reactions with several of the common alkaloid precipitating reagents. In addition, these compounds can react to produce similar results on chromatograms treated with the modified Dragendorff spray reagent. A critical analysis of the methods used to detect alkaloids in plant material can aid in the prevention, as well as in the interpretation of these false-positive reactions. If the Swanholm, Webb, or Kiang methods are to be utilized, heat should be avoided, or an additional purification step, as included in the Wall method, should be considered. The appearance of orange spots on chromatograms after treatment with the modified Dragendorff spray reagent should be

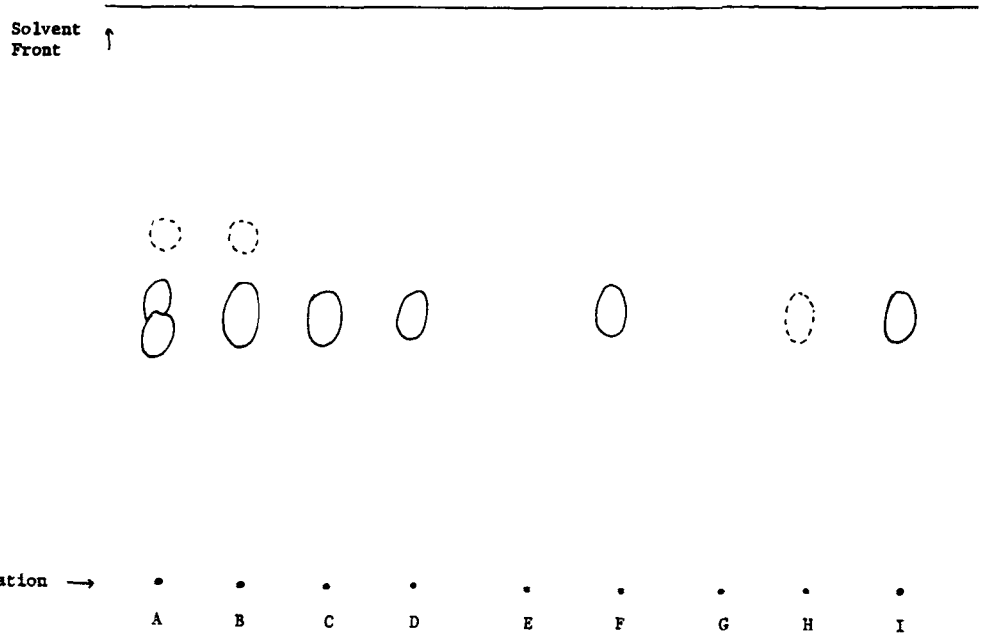


Fig. 3.—Thin-layer chromatogram of certain *P. methysticum* extracts, *P. methysticum*-treated control extracts, and control extracts. Key (after sulfuric acid spray): O = orange, P = pink, R = red, Y = yellow. Broken lines indicate trace concentrations. A, six known α-pyrones in chloroform; B, chloroform extract of *P. methysticum;* C, P. *methysticum* Prollius extract; D, *P. methysticum* Prollius extract + *H. virginiana;* E, *H. virginiana* Prollius extract; F, *P. methysticum* extract + *P. serotina;* G, *P. serotina* Prollius extract; H, *P. methysticum* extract + 1% HCl (unheated); I, *P. methysticum* extract + 1% HCl (heated).

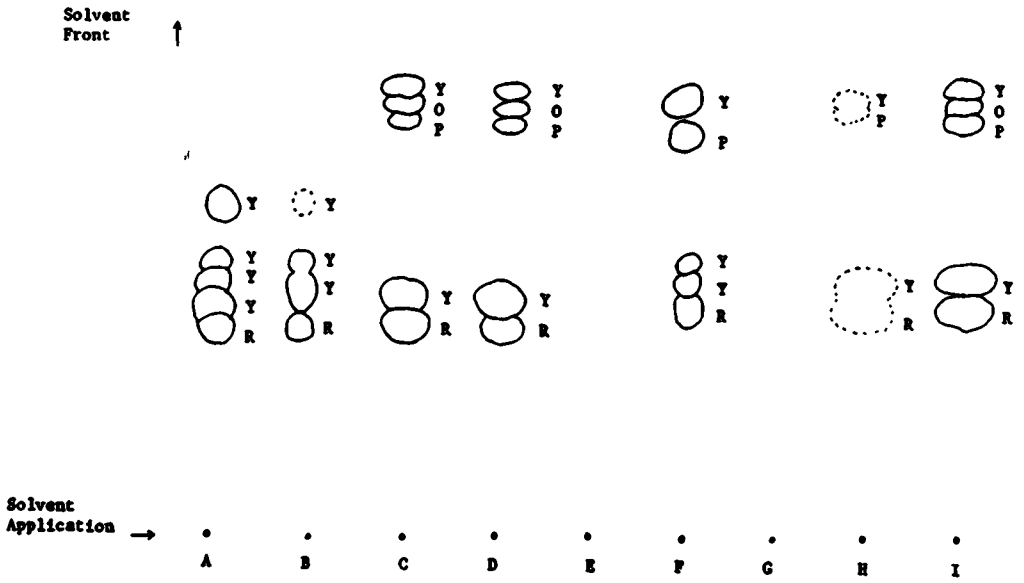


Fig. 4.—Thin-layer chromatogram of certain *P. methysticum* extracts, *P. methysticum* treated extracts, and control extracts following treatment with Dragendorff spray reagent. Circled areas indicate orange spots. Broken lines are indicative of trace concentrations. A, six known α-pyrones in chloroform; B, chloroform extract of *P. methysticum;* C, *P. methysticum* Prollius extract; D, *P. methysticum* Prollius extract + *H. virginiana;* E, *H. virginiana* Prollius extract; F, *P. methysticum* extract + *P. serotina;* G, *P. serotina* Prollius extract; H, *P. methysticum* extract + 1% HCl (unheated); I, *P. methysticum* extract + 1% HCl (heated).

considered indicative of the presence of alkaloids only when the suspected alkaloid spot persists for several hours, since compounds of the α-pyrone type, and perhaps others, will not retain the typical alkaloid-positive orange color with this reagent.

From the data presented, it is reasonable to assume that the occurrence of alkaloids in *P. methysticum*, as reported by several investigators, has been brought about through observations of the reactions due to the nonalkaloid α-pyrone constituents in this plant. These observations have prompted us to investigate other structurally related non-nitrogenous compounds, synthetic as well as naturally occurring, in order to gain additional information concerning the limitations of alkaloid detecting reagents as used in phytochemical studies. These results will be published at a later date.

SUMMARY

1. The nonalkaloid α-pyrone compounds present in *P. methysticum* have been shown to exhibit alkaloid-like reactions with several common alkaloid precipitating reagents.

2. The use of heat in alkaloid testing procedures increases the solubility of the α-pyrone compounds. This increased solubility is sufficient to allow detection of them with standard alkaloid reagents.

3. The α-pyrones present in *P. methysticum* will produce alkaloid-like color reactions on chromatograms treated with the Munier and Macheboeuf modified Dragendorff spray reagent. This effect is discussed and the sensitivity of the reaction on thin-layer chromatograms of the α-pyrones is reported.

4. The color reactions and sensitivity of kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin after thin-layer chromatography and subsequent sulfuric acid spray, is reported.

5. Alkaloids have been shown to be absent from *P. methysticum*. Previous reports concerning the presence of alkaloids in this plant have been evaluated and discussed in view of the experimental results from this investigation.

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Complex Formation Influence on Reaction Rate I

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A NUMBER of investigations have demonstrated that molecular complex formation can influence the rate at which participating species undergo chemical reaction. For example, the rate of the coupling reaction between β-naphthol and *p*-diazobenzenesulfonic acid was found by Overbeek, Vink, and Deenstra (1) to be lowered by the addition of caffeine to the reaction medium. The lowering of the reaction velocity was explained on the basis of lowered reactivities of the reactants in the complexed forms since both phenol and the sulfonic acid were shown to form 1:1 complexes with caffeine. Similarly, Higuchi, Lachman, Ravin, and Guttman (2,3,4) showed that the rates of hydrolysis of local anesthetic esters were substantially reduced by the addition of complexing agents to their aqueous solutions. Their investigations indicated that the esters, in the complexed forms, did not undergo hydrolytic cleavage at the ester linkage.

Pharmaceutical interest in this particular manifestation of complex formation is quite naturally derived from the possibility of utilizing such behavior to stabilize labile medicinal agents. More comprehensive studies of this phenomenon would, therefore, be of value in amplifying and extending this interest and could, in addition, be of more fundamental importance in providing further insights into the nature of the bonding forces responsible for molecular associations. The extent to which the reactivity of a compound is affected by complexation might serve as an indication of the type of interactive mechanism that is primarily operant. Correlation and comparison of such studies with results obtained by nonkinetic methods might also serve to pinpoint functional groups as sites of contact between components in the complex. Alternatively, it is possible that a kinetic approach to the determination of the energetics of complex formation might be more direct and preferred in some cases to many of the classical approaches which are commonly used.

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Accepted for publication March 15, 1962.

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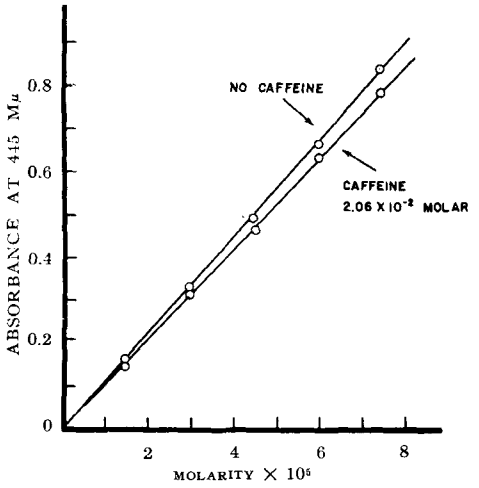


Fig. 1.—The relationship between absorbance and concentration for solutions of riboflavin.

lecular interactions on the velocities of reactions involving riboflavin. It is anticipated that riboflavin-containing systems will be useful models for the accumulation of pertinent information because of the well-known interactive tendencies of isoalloxazine derivatives and because the many modes of degradation and reaction of the vitamin will provide the opportunity to investigate the influences of complexation on the rates of reactions possessing quite different mechanisms. The specific objective of the present study was to investigate the influence of caffeine on the rate of the base-catalyzed conversion of riboflavin to urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline-carboxylic acid (5). No attempt was made to derive a mechanistic picture of the degradative reaction or a mechanistic explanation at the molecular level of the observed influence of complex formation on reactivity. The study will show, however, that the vitamin in its complexed form possessed negligible reactivity towards alkaline hydrolysis and that, consequently, pertinent information concerning energy changes which resulted from complexation could be obtained from kinetic studies.

EXPERIMENTAL[1]

Reaction systems were formulated in 0.05 *N* sodium hydroxide to contain approximately 1 × 10^{-4} moles $L.^{-1}$ of riboflavin and the desired concentration of caffeine. The reaction flasks were maintained at a constant temperature by immersion in a Sargent Thermonitor constant temperature bath. Five-milliliter samples were withdrawn from the flasks at various time intervals and immediately treated with exactly 5 ml. of 1 *N* acetic acid to quench the reaction. The absorbance of each sample was then determined at 445 mμ utilizing a Beckman model DU spectrophotometer. Studies were conducted in a similar manner on reaction systems containing 0.1 *N* sodium hydroxide. All riboflavin-containing solutions were protected from light at all times. The disappearance of riboflavin was thus followed by noting the decrease in absorbance at 445 mμ. The spectrophotometric assay appeared to be specific for the intact vitamin. Completely degraded solutions yielded negligible absorbance values at the selected wavelength. Although it has been shown by Harbury and Foley (6) that caffeine does affect the spectral characteristics of riboflavin in aqueous solution, preliminary studies which are illustrated in Fig. 1 showed that a direct proportionality existed between absorbance at 445 mμ and the concentration of the vitamin in both the absence and presence of caffeine. The absorbance values were, therefore, used directly in all of the kinetic plots as being representative of the vitamin concentrations.

RESULTS

Results typical of those obtained throughout the course of this study are graphically illustrated in Fig. 2. Here, semi-log plots are shown for two reaction systems formulated with and without caffeine. It can be seen that in both cases a pseudo first-order disappearance of the vitamin occurred. A number of studies showed that the linearity of the plots extended over time periods many times the half-life of the vitamin. Figure 2 makes apparent that the reaction occurred at a significantly reduced rate in the presence of caffeine. Similar studies were conducted at various temperatures and at various concentrations of caffeine. In all cases the data fit first-order plots and from such plots first-order rate constants

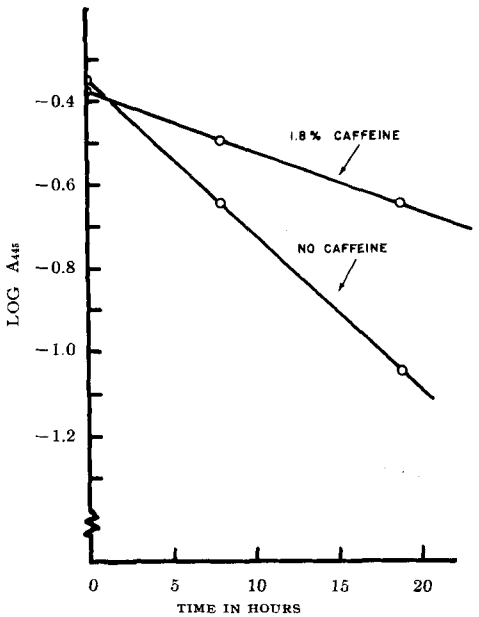


Fig. 2.—The first-order decomposition of riboflavin in 0.05 *N* sodium hydroxide at 35°.

[1] The author wishes to acknowledge the competent technical assistance of J. David McCallister.

were estimated. The results are summarized in Table I. Here, for each temperature investigated, are tabulated the molar concentration of caffeine in the system, the apparent rate constant observed for that system, and the ratio of the rate constant found in the absence of caffeine, k, to that found in the presence of caffeine, k_{app}. It can be seen that this ratio, which is indicative of the apparent reduction in vitamin reactivity in the presence of caffeine, is proportional to the caffeine concentration in the system.

DISCUSSION

A reasonable explanation for the decreased rate of vitamin disappearance effected by the presence of caffeine is that riboflavin in the complexed state possessed either a decreased or negligible reactivity toward the catalyst. If it is assumed that the latter was the case, then the overall mechanism for the process in the presence of caffeine is

$$\text{riboflavin:caffeine} = \text{caffeine} + \text{riboflavin}$$
$$\downarrow \text{OH}^-$$
$$\text{products}$$

Accordingly, the rate of disappearance will depend not on the stoichiometric concentration of riboflavin in the system but rather on the concentration of the noncomplexed specie.

If, as is indicated by the postulated mechanism, a 1:1 complex is formed, then it can be shown that the concentration of noncomplexed riboflavin is related to its stoichiometric concentration by the expression

$$R_f = \frac{K}{K + C_f} R_t \qquad \text{(Eq. 1)}$$

where R_f = molar concentration of noncomplexed riboflavin, K = dissociation constant for the complex, C_f = concentration of noncomplexed caffeine, and R_t = stoichiometric molar concentration of riboflavin. Since the rate equation for the decomposition reaction in the absence of caffeine is

$$-dR_t/dt = kR_t \qquad \text{(Eq. 2)}$$

then based on the preceding assumptions, in the presence of caffeine, it will be

$$-dR_t/dt = k \frac{K}{K + C_f} R_t \qquad \text{(Eq. 3)}$$

The apparent rate constants evaluated for reaction systems containing caffeine are thus related to the "true rate" constant by

$$k_{app} = k \frac{K}{K + C_f} \qquad \text{(Eq. 4)}$$

or more conveniently,

$$k/k_{app} = 1 + C_f/K \qquad \text{(Eq. 5)}$$

Because caffeine was maintained at a much higher concentration than riboflavin in all of the present studies, its stoichiometric concentration can be used as a close approximation of the C_f term in Eq. 5.

Equation 5 predicts a linear relationship between the ratio k/k_{app} and the molarity of caffeine and thus serves as a means to check the validity of the assumptions made. A plot of this relationship for three different temperatures is shown in Fig. 3. It is seen that, at least at lower concentrations of caffeine, a linear relationship existed. As the molarity of caffeine was increased, deviations from linearity became more pronounced. It is felt that this behavior does not negate the postulated mechanism but rather resulted from the self-complexing of caffeine which becomes increasingly significant at higher concentrations. This possibility is supported by the decreased curvature observed at higher temperatures and by the fact that a previous study on the solubilization of riboflavin by caffeine (7) yielded deviations of the same nature and of the same magnitude. It should be noted that the 35° curve of Fig. 3. includes experimental points obtained at two different concentrations of hydroxide ion. The close agreement obtained between the two different concentrations emphasizes that the stabilization did not result from neutralization or inactivation of catalyst by caffeine.

Dissociation constants for the complex were graphically estimated from the slopes of the curves obtained by the plot illustrated in Fig. 3. The initial straight-line portion of the curves were used for

TABLE I.—RATE CONSTANTS FOR THE BASE-CATALYZED DECOMPOSITION OF RIBOFLAVIN IN 0.05 N SODIUM HYDROXIDE IN THE ABSENCE AND PRESENCE OF CAFFEINE

°C.	Concn. Caffeine, mol./L. × 10^2	Rate Constant, $hr.^{-1}$ × 10^2	k/k_{app}
8	0	0.326	1.00
	0.778	0.244	1.34
	1.10	0.211	1.55
	2.36	0.171	1.91
	3.34	0.148	2.20
	3.94	0.138	2.36
	5.10	0.130	2.50
25	0	2.85	1.00
	0.472	2.42	1.18
	1.42	1.85	1.54
	2.34	1.69	1.69
	4.25	1.32	2.16
	6.60	1.15	2.48
30	0	5.25	1.00
	0.506	4.35	1.21
	1.44	3.50	1.50
	3.00	2.78	1.89
	4.24	2.39	2.20
	7.02	2.12	2.48
35	0	8.47	1.00
	0.943	6.60	1.28
	2.79	5.00	1.70
	4.76	4.11	2.06
	6.62	3.73	2.27
	8.50	3.26	2.52
35 (in 0.1 N NaOH)	0	13.8	1.00
	2.40	8.40	1.60
	7.20	5.68	2.43
50	0	33.8	1.00
	2.41	23.7	1.43
	4.70	20.2	1.84
	9.40	16.1	2.10
	14.2	15.5	2.18
70	0	198.	1.00
	2.40	151.	1.31
	4.70	138.	1.43
	9.40	110.	1.80
	11.8	104.	1.90
	14.2	109.	1.82
	16.6	96.0	2.06
	18.8	92.5	2.14

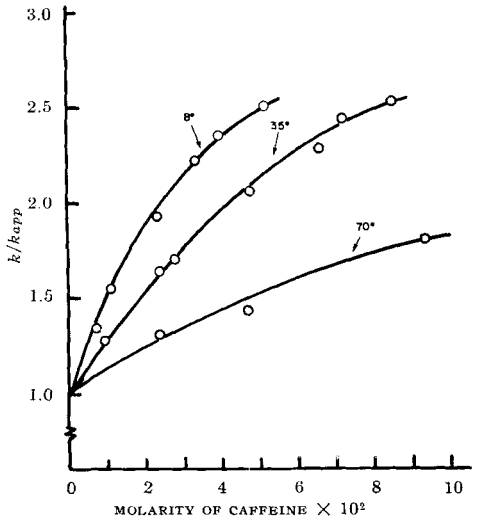


Fig. 3.—The effect of caffeine on the rate of decomposition of riboflavin in 0.05 *N* sodium hydroxide.

this purpose. The values obtained at various temperatures are tabulated in Table II.

The values obtained are in reasonable agreement with values derived by other workers who utilized different experimental methods. For example, the value for 20° was interpolated from this study to be 0.024 mol./L. as compared to a value of 0.019 mol./L. obtained by the spectrophotometric study of Yagi and Matsuoka (8) at the same temperature. Similarly, the solubility study at 30° of Guttman and Athalye (7) yielded a value of 0.0345 as compared to 0.0286 in the present study.

The temperature dependency of the dissociation equilibrium is graphically illustrated by the semi-log plot of Fig. 4. The enthalpy change $\Delta H°$ resulting from the association of caffeine with riboflavin was calculated to be −5.74 Kcal. The free energy change $\Delta F°$ and the entropy change $\Delta S°$ for the interaction at 35° were calculated to be −2.04 Kcal. and −12.0 e.u., respectively.

The temperature dependency for the degradative reaction, under the conditions used in this study, is shown by the Arrhenius plot of Fig. 5. Plotted here as a function of reciprocal absolute temperature are rate constants obtained at different temperatures for systems free of caffeine and also those obtained at corresponding temperatures for systems containing two different concentrations of caffeine. The apparent energy of activation was graphically estimated to be approximately 20.0 Kcal. in all cases. This observation gives further support to the assumption that the complexed form of riboflavin does not degrade at a perceptible rate.

This investigation has provided another example of a manifestation of complex formation that has possible practical and theoretical importance. In addition, the possibility of gaining information about the energetics of molecular interactions from kinetic studies has been demonstrated. Although quite equivocal, one might speculate that the reduction in the reactivity of riboflavin as well as the rather large

TABLE II.—DISSOCIATION CONSTANTS FOR THE RIBOFLAVIN-CAFFEINE COMPLEX

Temp., °C.	*K*, mol./L. × 10²
8	1.61
25	2.72
30	2.86
35	3.57
50	5.70
70	10.0

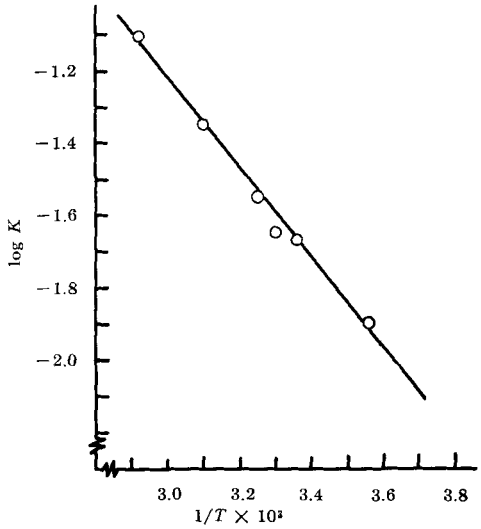


Fig. 4.—The temperature dependency of the interaction between riboflavin and caffeine.

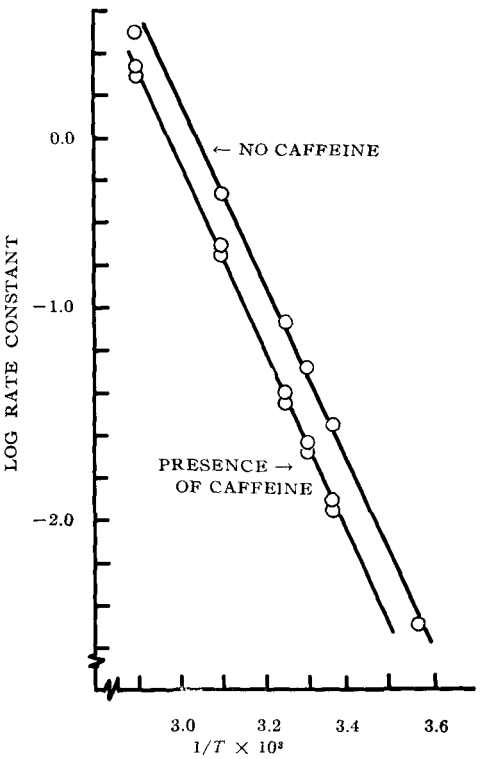


Fig. 5.—Arrhenius plot for the decomposition of riboflavin in 0.05 *N* sodium hydroxide

negative entropy change resulting from complexation supports the previously suggested involvement of a charge-transfer interactive mechanism with caffeine acting as a donor and riboflavin as the acceptor (6, 9). Such an interaction would possibly reduce the electrophilicity of riboflavin and might, therefore, reduce its reactivity by discouraging the nucleophilic attack of hydroxide ion, an attack undoubtedly involved in the hydrolytic cleavage of the isoalloxazine ring.

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Effect of Deuterium Oxide on Local Anesthetic Activity of Procaine

By S. V. SUSINA, F. D. HITER, F. P. SIEGEL, and M. I. BLAKE

Deuterium oxide was used as a solvent for procaine and the effect on the stability and local anesthetic activity was noted. The ED_{50} was determined by the method of Chance and Lobstein and comparison was made with aqueous procaine solutions. In water the ED_{50} was 1.8% while in deuterium oxide it was 1.0%. The LD_{50} was determined by intraperitoneal injection in mice. There appears to be no significant difference in the toxicity of procaine in either solvent. Stability studies indicate that procaine is more stable in deuterium oxide at pH and "apparent" pH values of 8.0, 8.5, and 9.0.

THE THERAPEUTIC efficacy of drugs instilled into the eye depends, among other factors, on the pH of the solution in which the drug is administered and on the stability of the drug under such conditions. Procaine is a typical example. These relationships may be adduced from kinetic data and pharmacological studies. Higuchi, *et al.* (1), have studied the influence of pH and temperature on the rate of procaine decomposition in aqueous solutions. It is apparent from their work that procaine deteriorates rapidly in the alkaline pH range. Inhibition of procaine decomposition by complex formation has also been studied (2).

Topical anesthetic activity of procaine depends primarily on the extent of availability of free base. At pH values below 7.0 procaine is relatively inactive topically. Hind and Goyan (3) have shown that at this pH the per cent of procaine free base is less than 1, and even at pH 7.4 it increases only to 3.4%, but at pH 9.0 it becomes 58.5%. The lipoid nature of the corneal epithelium controls the rate at which absorption of procaine will occur in the cornea. The greater the concentation of procaine free base in the lacrimal fluid, the greater should be the extent of procaine penetration and absorption from the cornea which should result in greater and more prolonged local anesthetic activity.

The present study was undertaken to determine the effect of deuterium oxide on the stability of procaine solutions at "apparent" pH values comparable to those in protium oxide (distilled water). In addition, comparison was made of the local anesthetic activity in both systems over the pH range 5.0 to 9.0.

The toxicity of deuterium oxide in biological systems was described by Morowitz and Brown (4). Katz (5) has reviewed the literature dealing with the chemistry and biology of deuterium oxide. Algae and other microorganisms have been cultured in 99.6% deuterium oxide (6). It has been demonstrated (5) that mice and rats can survive when up to about one-third of their body water is replaced by deuterium oxide. When the blood serum approaches about 20% deuterium oxide content, toxic manifestations become evident. Since it appears that deuterium oxide is toxic in animals only when the concentration

Received March 27, 1962, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago.

Accepted for publication June 26, 1962.

The authors express their gratitude to the University of Illinois Research Board for financial support in this project.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

This paper is abstracted in part from a dissertation submitted to the Graduate College of the University of Illinois at the Medical Center by Franklin D. Hiter in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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Effect of Deuterium Oxide on Local Anesthetic Activity of Procaine

By S. V. SUSINA, F. D. HITER, F. P. SIEGEL, and M. I. BLAKE

Deuterium oxide was used as a solvent for procaine and the effect on the stability and local anesthetic activity was noted. The ED_{50} was determined by the method of Chance and Lobstein and comparison was made with aqueous procaine solutions. In water the ED_{50} was 1.8% while in deuterium oxide it was 1.0%. The LD_{50} was determined by intraperitoneal injection in mice. There appears to be no significant difference in the toxicity of procaine in either solvent. Stability studies indicate that procaine is more stable in deuterium oxide at pH and "apparent" pH values of 8.0, 8.5, and 9.0.

THE THERAPEUTIC efficacy of drugs instilled into the eye depends, among other factors, on the pH of the solution in which the drug is administered and on the stability of the drug under such conditions. Procaine is a typical example. These relationships may be adduced from kinetic data and pharmacological studies. Higuchi, *et al.* (1), have studied the influence of pH and temperature on the rate of procaine decomposition in aqueous solutions. It is apparent from their work that procaine deteriorates rapidly in the alkaline pH range. Inhibition of procaine decomposition by complex formation has also been studied (2).

Topical anesthetic activity of procaine depends primarily on the extent of availability of free base. At pH values below 7.0 procaine is relatively inactive topically. Hind and Goyan (3) have shown that at this pH the per cent of procaine free base is less than 1, and even at pH 7.4 it increases only to 3.4%, but at pH 9.0 it becomes 58.5%. The lipoid nature of the corneal epithelium controls the rate at which absorption of procaine will occur in the cornea. The greater the concentation of procaine free base in the lacrimal fluid, the greater should be the extent of procaine penetration and absorption from the cornea which should result in greater and more prolonged local anesthetic activity.

The present study was undertaken to determine the effect of deuterium oxide on the stability of procaine solutions at "apparent" pH values comparable to those in protium oxide (distilled water). In addition, comparison was made of the local anesthetic activity in both systems over the pH range 5.0 to 9.0.

The toxicity of deuterium oxide in biological systems was described by Morowitz and Brown (4). Katz (5) has reviewed the literature dealing with the chemistry and biology of deuterium oxide. Algae and other microorganisms have been cultured in 99.6% deuterium oxide (6). It has been demonstrated (5) that mice and rats can survive when up to about one-third of their body water is replaced by deuterium oxide. When the blood serum approaches about 20% deuterium oxide content, toxic manifestations become evident. Since it appears that deuterium oxide is toxic in animals only when the concentration

Received March 27, 1962, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago.

Accepted for publication June 26, 1962.

The authors express their gratitude to the University of Illinois Research Board for financial support in this project.

Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.

This paper is abstracted in part from a dissertation submitted to the Graduate College of the University of Illinois at the Medical Center by Franklin D. Hiter in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

in the body fluids reaches high levels, and since deuterium oxide resembles the common solvent protium oxide more closely than any other, it seems appropriate to explore possible pharmaceutical applications of this solvent.

EXPERIMENTAL

Toxicity Study.—The effect of deuterium oxide on the toxicity of procaine was studied by determining the LD_{50} in this solvent. This was conducted on BDF-1[1] strain mice weighing about 25 Gm. Two per cent procaine hydrochloride solutions were prepared in water and deuterium oxide. The doses administered varied from 165 mg./Kg. to 200 mg./Kg. The route of administration was by intraperitoneal injection and each of the four dose levels was administered to a group of 10 mice. See Table I. The LD_{50} was determined by use of the method of Miller and Tainter (7).

TABLE I.—TOXICITY OF PROCAINE IN WATER AND IN DEUTERIUM OXIDE

Procaine in Water		Procaine in Deuterium Oxide	
Dose, mg./Kg.	Death, %	Dose, mg./Kg.	Death, %
165	20	170	10
175	30	180	40
190	50	190	50
200	60	200	60

Determination of ED_{50}.—The cornea provides an ideal site for testing surface anesthesia. It is free of special sensory cells and the free, unsheathed nerve endings are imbedded in the corneal layers. Therefore, the drug has a single uniform membrane to penetrate after which it is in direct contact with sensory nerve fibers. The cornea is also free of fluid channels and blood vessels. It has been shown (8) that the guinea pig elicits a more regular reflex response than the rabbit. In this investigation a modification of the method of Chance and Lobstein (8) was employed.

The local anesthetic potency of procaine in water and deuterium oxide was determined on the cornea of guinea pigs weighing 225–250 Gm. Each animal was held immobile in plastic restraining cages which allowed easy access to the eyes for the instillation of solution. Aqueous procaine solutions were freshly prepared prior to use by dissolving procaine hydrochloride in 0.1 *M* phosphate buffer (pH 7.0) in concentrations ranging from 0.5 to 2.0%. The concentrations of procaine in deuterium oxide ranged from 0.25 to 1.0% and were prepared in a deuterated phosphate buffer solution (pD 7.4). Two drops of the solution were placed on the cornea filling the space between the eyelids. The resulting clearly visible film of solution was allowed to remain in contact with the cornea for 15 seconds. After this period the excess fluid was removed by initiating a blink reflex. At the end of 45 seconds, a stimulus was applied to the cornea of the eye by means of a single thick horsehair which was pressed against the center of the cornea. The same amount of pressure was applied with each stimulation. This was repeated at 1-minute intervals for a period of 1 hour. A failure to respond to the stimulus (the blink reflex) indicated effective anesthesia. The anesthetic effect, recorded as per cent protection, was calculated from the number of minutes in which the blink reflex did not occur out of the 60-minute study period. A minimum of 12 animals or 24 test sites was used for each test solution and the reported value, expressed as a percentage, is the average value for that number of determinations. The logarithm of the concentration of procaine was plotted *vs.* the probit of the per cent protection (Fig. 1). The concentration of procaine solution which produced 50% protection was then determined from the curve. This value is the effective $dose_{50}$ (ED_{50}).

Effect of pH and pD on Anesthetic Activity.—One per cent procaine solutions were buffered at pH 5.0, 6.0, 7.0, 8.0, and 9.0. One-tenth molar buffer solutions were prepared with KH_2PO_4 and sodium hydroxide for pH values 5.0 to 8.0. A sodium borate and hydrochloric acid buffer system was used for pH 9.0. The concentration of procaine in deuterium oxide was 1/2%. The deuterium oxide buffer systems were prepared from the same reagents except that all replaceable hydrogen was exchanged with deuterium. All buffers in deuterium oxide were adjusted to an "apparent" pH corresponding to the pH of the aqueous buffer solutions. The "apparent" pH was measured with a Beckman pH-meter (model H-2) and an appropriate correction (9) to the reading was made to obtain pD.

The corneal reflex test described earlier was used to determine the per cent protection at the different pH and pD values. The data are shown graphically in Fig. 2.

Procaine Stability Study.—The decomposition of procaine in protium oxide and deuterium oxide solutions was studied by the method described by Higuchi, *et al.* (1). The time required for procaine to decompose to 50% of the original concentration (half-life) was determined at pH 8.0, 8.5, and 9.0

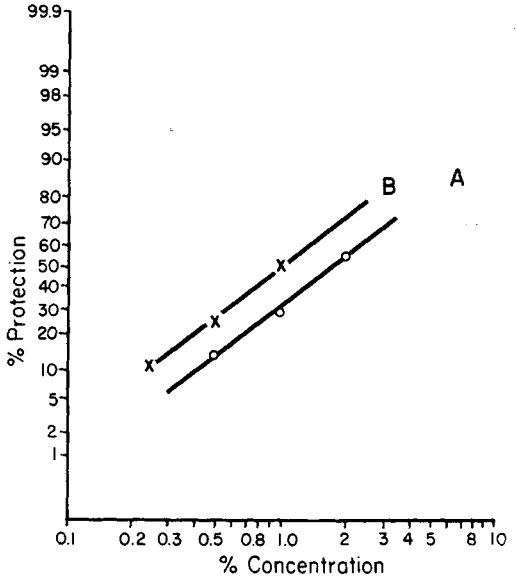


Fig. 1.—Per cent protection plotted in probits; concentration of procaine plotted as logarithm. Curve *A*, procaine in water; curve *B*, procaine in deuterium oxide.

[1] Simonsen Laboratories, White Bear, Minn.

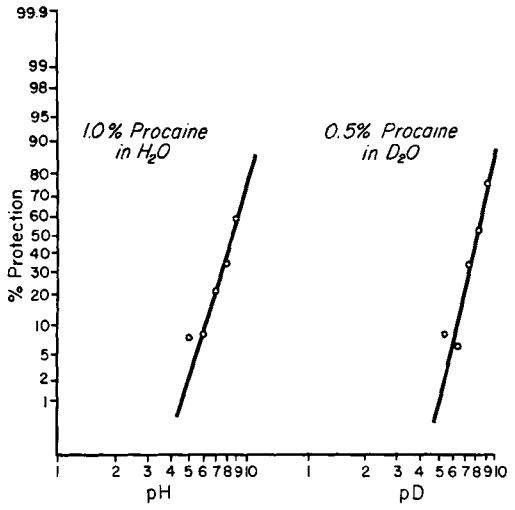


Fig. 2.—Effect of pH and pD on local anesthetic effect of procaine. Per cent protection plotted in probits; pH and pD plotted as logarithm.

in aqueous buffer solutions and at corresponding pD values for the deuterated system. The replaceable hydrogens in procaine hydrochloride were not exchanged with deuterium prior to the study in the deuterium oxide solutions. The hydrogen introduced in this way did not appreciably affect the hydrogen content of the deuterium oxide. This was verified by analyzing the reaction mixture for deuterium content. The data for these studies are recorded in Table II.

TABLE II.—COMPARISON OF THE RATE OF HYDROLYSIS OF PROCAINE IN PROTIUM OXIDE AND DEUTERIUM OXIDE AT 40°C.

Protium Oxide System		Deuterium Oxide System		
pH	Half-life, hr.	pD	Half-life, hr.	$T^1/_2\ D_2O^b / T^1/_2\ H_2O$
8.0	38	8.4 (8.0)[a]	115	3.0
8.5	13	8.9 (8.5)	38	2.9
9.0	6.5	9.4 (9.0)	13	2.0

[a] "Apparent" pH. [b] Ratio of half-lives at "apparent" pH in D_2O and pH in H_2O.

RESULTS AND DISCUSSION

The LD_{50} for procaine hydrochloride in water was 190 ± 12 mg./Kg. and for procaine in deuterium oxide was 190 ± 8 mg./Kg. The data for the LD_{50} studies are shown in Table I. There does not appear to be any significant difference in the toxicity of procaine in either solvent system as determined by intraperitoneal injection in mice.

An appropriate dose of procaine which produced a measurable local anesthetic effect was obtained from a dose response curve (Fig. 1) for procaine in both protium and deuterium oxide solutions. The ED_{50} for procaine in water (curve *A*) is 1.8% ± 0.8%. For procaine in deuterium oxide (curve *B*) the ED_{50} is 1.0% ± 0.5%. The pH of the aqueous procaine solution was 7.0 and that of the procaine in deuterium oxide solution was an "apparent" pH of 7.0 or a pD of 7.4. It appears that the ED_{50} of procaine in deuterium oxide is about one-half of the ED_{50} for procaine in water.

In the study dealing with the effect of pH and pD on the local anesthetic activity concentrations of 1.0% procaine in water and 0.5% procaine in deuterium oxide were used. Per cent protection as a function of pH and pD is shown graphically in Fig. 2. At pH values below 7.0 the per cent protection was low. This in agreement with the findings of Hind and Goyan (3) and Swan (10) who showed that at a pH below 7.0 the per cent of free base is less than 1%. Poor anesthetic activity is expected since it is only the free base which is capable of producing this effect. As the pH is increased to 7.5 the per cent protection increased to 30% and at pH values of 8.0 and 9.0 it increased to 35% and 60%, respectively. At pD 7.4 the per cent protection was 32% and at pD 8.4 and 9.4 the protection increased to 51% and 75%, respectively. At comparable pH and "apparent" pH values the deuterium oxide system offers greater protection than an aqueous system. While per cent protection was approximately the same, the concentration of procaine in deuterium oxide was one-half of that in the aqueous system.

The stability studies indicate that procaine is more stable in deuterium oxide solutions as compared to aqueous solutions at the same pH and "apparent" pH. Increased local anesthetic activity of procaine in deuterium oxide may be accounted for by the fact that procaine free base is more stable in deuterium oxide solutions or that at the same pH and "apparent" pH, a deuterium oxide solution of procaine will contain more free base than in the corresponding aqueous solution.

A simple pH effect is ruled out by the fact that much higher ratios of half-lives are obtained when pH and pD are compared. For example, when aqueous procaine at pH 8.0 is compared with procaine in deuterium oxide at "apparent" pH 8.0, the ratio of half-lives is 3.0. See Table II. However, when an aqueous solution of procaine at pH 8.5 is compared to procaine in deuterium oxide at pD 8.4, the ratio $T^1/_2\ D_2O / T^1/_2\ H_2O$ becomes 8.8.

The availability of deuterium oxide at a reasonable cost has stimulated much research with this solvent in recent years. However, a review of the literature indicated that pharmaceutical applications of this solvent have not been explored. It was the purpose of this investigation to observe the effect of deuterium oxide on the stability of procaine in alkaline solutions and to note the influence on local anesthetic activity. Further studies are currently in progress in which other labile drugs are being investigated. In addition to the effect of this solvent on the stability and activity of certain drugs, it is hoped that information may be obtained that will better explain the mechanisms of drug action and decomposition.

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Chromatography and Electrophoresis of Phenothiazine Drugs

By THEODORE J. MELLINGER and CLYDE E. KEELER

Three separation techniques for various phenothiazine tranquilizers are reported in this paper. Electrophoresis with suitable buffer systems and proper voltage permitted fast and distinctive migration of these phenothiazine bases, but tailing interfered with the usefulness of this technique. Paper chromatography with salt solutions as solvents proved to be a valuable and easy procedure for the separation of this group of drugs. Thin-layer chromatography with silica gel showed the most accurate separation of phenothiazine compounds. Combination of chromatography with color reaction or fluorescence permitted good differentiation of the various phenothiazine tranquilizers.

INCREASING INTEREST in the therapy of mental disorders has led to widespread use of phenothiazine derivatives, that are on the market under various names and chemical modifications. There is an immense clinical literature concerning these drugs but little is known about their separation or identification by means of chromatographic procedures or electrophoresis. Three kinds of techniques have been explored and are reported in this paper: electrophoresis, paper chromatography, and thin-layer chromatography. They were tested upon 23 drugs in the search for well reproducible separation methods with variation in R_f values as well as distinctive spots or zones devoid of tailing.

METHODS

Electrophoresis was carried out with buffer systems proposed by Werum, *et al.* (1). The 23 drugs were applied side by side along the starting line on a strip of Whatman 3 MM paper 30 cm. in width. Several strips were run with each buffer system. A Gordon-Misco apparatus for horizontal paper electrophoresis was used. The average potential difference between the electrodes was 500 to 800 v.

Paper chromatography was carried out with Whatman 3 MM filter paper. The dissolved substances were applied in amounts of 5 to 10 mg. in thin streaks along the starting line and were developed in the ascending direction.

Thin-layer chromatography was run on glass plates 20 × 20 cm. coated with a layer of 500 μ of silica gel G Merck. The coated plates were dried overnight in an incubator at 38°. After spotting the drugs, the chromatograms were developed in the ascending direction.

To locate the drugs, their fluorescence was examined under an ultraviolet lamp with a peak emission at 253 mμ and one at 360 mμ. To produce color reactions, 40% sulfuric acid was sprayed upon the developed chromatograms.

RESULTS

Electrophoresis.—Because all phenothiazine drugs in this study are bases, and therefore positively charged molecules, they migrate toward the negative end of the paper. With appropriate buffer systems and a proper potential difference between the two poles, migration of the drugs was accomplished in 40 to 60 minutes. With the new organic buffers proposed by Werum, *et al.*, the migrating phenothiazine compounds could be followed easily by their fluorescence under ultraviolet light. These new buffers do not quench the fluorescence of the drugs as barbiturate buffers do. When the buffer solutions were modified by leaving out the formamide, the same migration was obtained and the color reactions were more distinctive.

Table I presents the electrophoretic values of 19 phenothiazine compounds and of four other substances closely related to the phenothiazine tranquilizers. The migration values of the drugs are shown in the vertical columns, as they were run on the same paper. However, the values for each substance cannot be compared exactly on the horizontal lines, since they were not run together. In spite of this, there can be seen a general trend in most of the substances to decrease the speed of migration with increasing pH, possibly due to decreasing ionization at higher pH. This is less apparent for the three sulfoxidized phenothiazines, imipramine, and the two thypendyl compounds. It cannot be ascertained from these experiments whether the deviations of the latter compounds are merely due to physico-chemical differences, or are

Received March 12, 1962, from the Research Division, Milledgeville State Hospital, Milledgeville, Ga.
Accepted for publication April 23, 1962.

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TABLE I.—PAPER ELECTROPHORESIS OF 19 PHENOTHIAZINE COMPOUNDS AND FOUR PHENOTHIAZINE-LIKE DRUGS AT DIFFERENT pH (MIGRATION IN CM., COMPARABLE VERTICALLY)

	pH					
	3.3	4.7	6.0	7.2	8.0	9.3
Chlorpromazine	5.4	6.2	5.2	4.9	5.4	1.8
Perphenazine	6.0	5.4	4.4	4.3	3.9	2.5
Prochlorperazine	6.4	5.5	4.6	4.0	3.8	1.8
Pipamazine	5.0	4.9	4.6	3.3	5.2	2.4
Thiopropazate	5.1	4.9	4.0	4.1	2.3	1.7
Acetophenazine	5.7	5.0	4.6	4.4	5.1	2.8
Proketazine	5.5	4.2	4.3	4.2	4.5	4.2
Butyrylperazine	5.8	4.2	3.6	2.8	3.3	4.1
Fluphenazine	6.8	3.7	4.3	4.5	2.7	1.2
Trifluoperazine	7.0	4.4	4.7	4.7	3.2	1.7
Triflupromazine	5.9	4.8	5.3	5.5	4.6	1.8
Promazine	6.5	7.0	6.7	6.0	7.7	7.5
Mepazine	6.0	6.3	5.6	5.2	6.2	4.6
Methoxypromazine	6.1	6.3	6.1	5.4	7.3	5.9
Thioridazine	4.9	4.5	5.4	4.6	4.1	1.9
Thiethylperazine	4.2	6.0	4.9	4.1	1.9	1.5
Chlorpromazine-sulfoxide	6.4	7.1	6.1	6.3	8.2	8.3
Thioridazine-sulfoxide	5.7	6.0	5.9	5.2	7.1	6.6
Trifluoperazine-sulfoxide	7.2	6.2	5.8	5.4	7.4	5.9
Phenothiazine-like drugs:						
Imipramine	6.4	6.9	6.1	5.8	8.7	7.3
Chlorprothixine	5.5	5.3	5.2	4.2	1.1	1.7
Isothypendyl	6.4	6.7	7.1	6.3	8.4	7.1
Prothypendyl	6.0	6.2	5.8	5.5	7.9	7.4

the result of chemical interaction with some of the organic buffer substances used.

With most buffer systems, the drugs migrated at very similar speeds when compared vertically in Table I. Buffer pH 8 permitted the best separation. A drawback of paper electrophoresis was excessive tailing of most compounds, except phenothiazine sulfoxides, when concentrations over 10 mg. per spot were applied.

Paper Chromatography.—When the conventional solvent systems of lower alcohols with water or acidic or basic solutions are used, all phenothiazine drugs run with or near the front, and form tails. Low affinity of the drugs for the stationary phase is probably the cause. Although this paper chromatographic technique is so unsatisfactory, it is the most frequently cited.

When the lipid solvents were omitted and salt solutions were used as solvents, the picture changed, and a satisfactory separation of all phenothiazine drugs could be carried out on paper chromatography. Small amounts of acids or lower alcohols added to these salt mixtures, not exceeding 10% of the total volume, permitted a further variation of R_f values. Table II shows the solvents used and the wide variety of R_f values for the phenothiazine compounds. When these solvent systems were studied, they appeared to be similar to "reverse phase techniques" since substances with higher lipid solubility showed lower R_f values.

Another method using mineral oil treated paper with methanol as the solvent gave very satisfactory separations without tailing, especially when used in circular chromatography to distinguish the original phenothiazine drugs from their metabolites in urine or bile. When the differentiation of one original phenothiazine derivative from another is concerned, it was not as suitable since the drugs developed nearly the same R_f of about 0.60 to 0.70.

Thin-layer Chromatography.—Although several techniques for the separation of lipids similar to paper chromatographic procedures had been described before (2–4), the introduction of thin-layer chromatography by Stahl (5–7), constituted real progress in the separation of lipid soluble substances. Reproducible standard conditions of a uniformly distributed layer of the absorbent is attained. Several substances or mixtures together with reference compounds can be developed and compared on the same chromatogram. We tested this technique for the separation of phenothiazine drugs that are all nitrogenous bases with relatively high fat solubility resembling, in this property, many of the alkaloids, antihistaminics, and synthetic narcotics.

When silica gel G Merck was used as the thin layer, all phenothiazine derivatives showed a strong absorption to silica gel. Chloroform, ether, hexane, or other lipid solvents did not move the substances from their starting line even after many hours of running the chromatogram. However, when an appropriate mixture of lipid solvent and water was used, the drugs migrated with a variety of quite distinctive R_f values. This is shown in Table III. The average time of development required in the ascending direction was 2 hours. This separation method was superior to that obtained by paper chromatography with salt solutions where a spreading out of the spots often takes place. The sensitivity or detectability of the spots moving in sharply defined zones was increased. Figure 1 shows the sharp separation of a thin-layer chromatogram compared with the spots of electrophoresis. Even when some of the drugs have nearly similar R_f values with one solvent system they can be distinguished by their fluorescences, their color reactions, or by a two-dimensional chromatogram. This property makes the method especially suitable for testing the purity or decomposition of phenothiazine drugs. The silica gel thin-layer technique proved to be, by far, the best procedure for any phenothiazine drug.

Location of the Drugs.—Since all phenothiazine

TABLE II.—R_f VALUES OF 19 PHENOTHIAZINE COMPOUNDS AND FOUR PHENOTHIAZINE-LIKE DRUGS WITH PAPER CHROMATOGRAPHY (NUMBERS REPRESENT $R_f \times 100$)

	1*N* Sodium formate	1*N* Sodium formate 90 *n*-Propanol 10	1*N* Sodium formate 90 1*N* Ammonia 10	1*N* Sodium formate 97 90% Formic acid 3	1*N* Sodium acetate	1*N* Sodium acetate 90 *n*-Propanol 10	10% Sodium chloride 92 *n*-Propanol 8
Time in minutes, ascending	40″	50″	110″	35″	30″	120″	100″
Chlorpromazine	34	32	11	43	30	54	70
Perphenazine	31	41	17	52	25	49	36
Prochlorperazine	18	20	11	58	18	43	44
Pipamazine	35	48	13	40	27	51	56
Thiopropazate	20	14	12	56	09	28	53
Acetophenazine	26	50	29	50	22	65	71
Proketazine	34	56	28	53	33	60	61
Butyrylperazine	19	35	18	42	17	48	45
Fluphenazine	23	32	19	51	15	45	47
Trifluoperazine	27	25	25	60	17	35	55
Triflupromazine	41	50	12	59	35	59	76
Promazine	58	61	20	63	56	88	80
Mepazine	41	50	13	53	38	69	71
Methoxypromazine	44	55	17	53	42	69	70
Thioridazine	24	40	09	37	24	45	52
Thiethylperazine	14	23	37	45	13	33	28
Chlorpromazine-sulfoxide	69	77	67	70	67	89	85
Thioridazine-sulfoxide	56	64	46	62	54	78	76
Trifluoperazine-sulfoxide	74	78	70	80	70	96	91
Phenothiazine-like drugs:							
Imipramine	65	78	19	68	62	84	83
Chlorprothixine	26	31	12	41	23	40	28
Isothypendyl	61	67	35	67	60	82	80
Prothypendyl	54	61	28	60	52	75	74

TABLE III.—R_f VALUES AND SOLVENT SYSTEMS FOR THIN-LAYER CHROMATOGRAPHY WITH SILICA GEL FOR 23 PHENOTHIAZINE DRUGS OR SIMILAR COMPOUNDS (NUMBERS REPRESENT $R_f \times 100$)

	tert-Butyl Alcohol 90 1*N* Ammonia 10	*n*-Propanol 88 1*N* Ammonia 12	Ether, Satd. with Water, Overrun	70% Methanol	85% *n*-Propanol	*n*-Butanol, Satd. with 1*N* Ammonia
Chlorpromazine	37	44	28	14	23	66
Perphenazine	28	57	07	48	24	53
Prochlorperazine	10	31	06	24	08	55
Pipamazine	38	71	..	41	37	79
Thiopropazine	64	79	49	65	53	81
Acetophenazine	12	36	04	52	18	38
Proketazine	19	45	06	53	25	44
Butyrylperazine	08	31	06	28	10	45
Fluphenazine	37	56	09	68	27	57
Trifluoperazine	18	33	09	34	12	51
Triflupromazine	36	50	48	22	22	72
Promazine	16	31	12	11	11	50
Mepazine	29	46	13	13	13	62
Methoxypromazine	15	26	12	12	09	45
Thioridazine	24	39	14	24	15	64
Thietylperazine	14	50	..	23	13	61
Chlorpromazine-sulfoxide	05	10	01	09	05	27
Thioridazine-sulfoxide	03	13	01	09	03	33
Trifluoperazine-sulfoxide	03	11	02	17	03	31
Phenothiazine-like drugs:						
Imipramine	45	55	24	13	16	66
Chlorprothixine	34	78	..	20	36	88
Isothypendyl	26	39	24	15	11	61
Prothypendyl	16	21	11	11	08	44

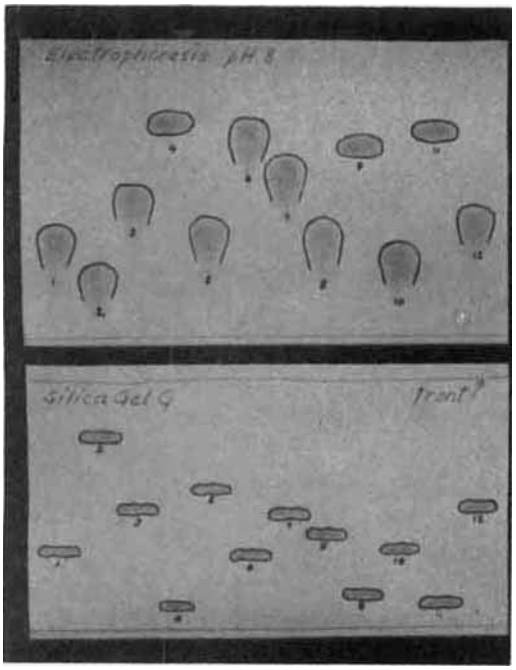


Fig. 1.—Comparable migration with paper electrophoresis buffer pH 8 (above), and thin-layer chromatography with silica gel, *n*-propanol 88 and 1 *N* ammonia 12 (below). 1, prochlorperazine; 2, thiopropazate; 3, chlorpromazine; 4, chlorpromazine-sulfoxide; 5, perphenazine; 6, promazine; 7, mepazine; 8, thioridazine; 9, thioridazine-sulfoxide; 10, trifluoperazine; 11, trifluoperazine-sulfoxide; 12, triflupromazine.

derivatives of this study showed a visible fluorescence of varying intensity under ultraviolet irradiation, this locating method was very satisfactory. An ultraviolet lamp having a maximum intensity at 263 mμ was superior to one with 360 mμ for this purpose. The sensitivity of this procedure was in many cases higher than with color reactions. Compounds having a chlorine on position 2 of the phenothiazine nucleus, such as chlorpromazine, perphenazine or perchlorperazine, or agents without substitution on this position, such as promazine and mepazine showed the least fluorescence or appeared often as quenched zones on the slightly fluorescent background of the paper or silica gel. This happened especially in solvent systems containing acids. It was found that the combination of acid solvent and ultraviolet or daylight easily decomposed the phenothiazine drugs, usually decreasing their visible fluorescence. Acidic solvent systems had to be used in light-protected tanks and avoided whenever possible.

When the phenothiazine drugs were run together on the same chromatogram, it could be easily observed that each emitted a fluorescent color according to its substitution on position 2 on the phenothiazine nucleus. For instance, drugs with a trifluomethyl group, as triflupromazine, trifluoperazine, or fluphenazine, emit the same bluish-yellow fluorescence. They could not be differentiated from each other by this method, but they could be distinguished easily from acylated compounds like acetophenazine, proketazine, or butyrylperazine, that showed an orange-to-brick-red fluorescence. Thioridazine showed a blue fluorescence which was strongly intensified by potassium permanganate spray. The fluorescent colors of the various phenothiazine drugs are shown in Table IV together with their color reactions.

All phenothiazine drugs responded to 40% sulfuric acid spray with various color reactions that distinguish them according to their radicals on position 2. As with the fluorescence, this color test did not permit any further differentiation between drugs with different radicals on position 10. When ferric chloride or ferric nitrate was added to 40% sulfuric acid spray, no difference was observed in color variation or intensity except the velocity of color development. These catalysts are useful in colorimetry of phenothiazines but superfluous in spray reagents for chromatograms. Adding 1% *p*-dimethylaminobenzaldehyde in 25% sulfuric acid did not show any further color difference, but later

Table IV.—Locating Methods for Phenothiazine Compounds and Phenothiazine-Like Drugs after Chromatography or Electrophoresis

	Visible Color After 40% H_2SO_4 Spray	Fluorescence Under U.V. Light
Chlorpromazine	violet	quenching[a] or faint blue
Perphenazine	violet	quenching or faint blue
Prochlorperazine	violet	quenching or faint blue
Pipamazine	violet	quenching or faint blue
Thiopropazate	violet	quenching or faint blue
Acetophenazine	orange-pink	orange-brick red
Proketazine	orange-pink	orange-brick red
Butyrylperazine	orange-pink	orange-brick red
Fluphenazine	orange	bluish-yellow
Trifluoperazine	orange	bluish-yellow
Triflupromazine	orange	bluish-yellow
Promazine	orange-pink	quenching or faint blue
Mepazine	orange-pink	quenching or faint blue
Methoxypromazine	purple-blue	quenching or faint blue
Thioridazine	blue	blue
Thiethylperazine	blue	blue
Chlorpromazine-sulfoxide	violet-pink	quenching
Thioridazine-sulfoxide	blue	blue
Trifluoperazine-sulfoxide	orange	blue
Phenothiazine-like drugs:		
Imipramine		quenching or faint blue[c]
Chlorprothixine	orange	[d]
Isothypendyl	yellow	bluish-yellow
Prothypendyl	yellow	bluish-yellow

[a] Dark zone on slightly fluorescent background. [b] Visible blue after adding $KMnO_4$ or bromine. [c] Increased blue-green fluorescence after 40% H_2SO_4 spray. [d] Brick fluorescence after 40% H_2SO_4 spray.

the spots turned to a green-blue tint. The palladium chloride reaction (8), despite its good sensitivity, showed the least color differentiation.

COMMENTS

The described procedures have revealed that the phenothiazine compounds examined in our experiments cannot be recognized completely by their color reactions or fluorescent properties. Only chromatography and electrophoresis made it possible to distinguish between substances giving the same color reactions or fluorescence but with a different radical on position 10 of the phenothiazine nucleus. This combination of chromatographic techniques with color reactions was of great help in many analyses in our laboratory concerned with the identification of phenothiazine drugs in toxicological investigations, in drug metabolism studies, in purification procedures, and in purity tests of commercial preparations.

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New 3-Thiophene Derivatives as Sedative Agents

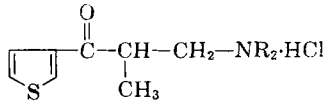
By HEINO A. LUTS† and W. LEWIS NOBLES

A group of compounds prepared from 3-propionylthiophene has been synthesized for pharmacological evaluation. Preliminary studies indicate that some of these agents combine sedative and stimulating properties in an unusual manner.

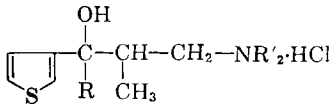
IN A SEARCH for compounds possessing possible analgetic activity, a program designed to produce certain Mannich bases and their derivatives from 3-propionylthiophene has been carried out. It has been noted that in many cases the 3-thienyl analog often has greater activity than the corresponding 2-thienyl compound (1–5). In fact, Campaigne (5) has suggested that, based on available data, it is impossible to predict the activity of the 3-thienyl isomer from a knowledge of the activity of the corresponding 2-isomer. Furthermore, Campaigne suggests that in any program in medicinal chemistry involving thiophene analogs, the 3-isomer should always be included since there is a high probability that it will be active and a good chance that it will be more active than the 2-isomer.

Previously, we (6) reported on the preparation of 2-substituted analogs of *d*-propoxyphene. In line with this and the above statements of Campaigne, it appeared quite logical to extend this work to the preparation of the corresponding 3-isomers.

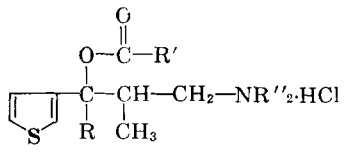
In our work, three general types of compounds were prepared. These are



Type I



Type II



Type III

The compound designated as Type I was prepared using the standard conditions normally employed in the Mannich reaction (7). Type II compounds were formed by the conventional Grignard reaction on the preceding Mannich base; also, the Mannich base was reduced to the

Received March 27, 1962, from the School of Pharmacy, University of Mississippi, University.
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Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
† Present address: Structure-Activity Research, Inc., College Hill, Oxford, Miss.

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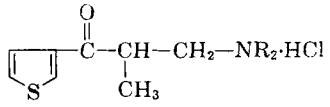
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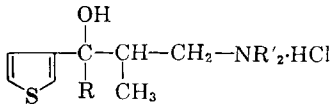
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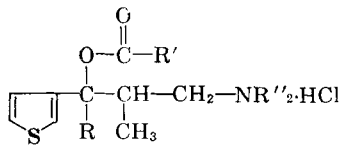
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Type III

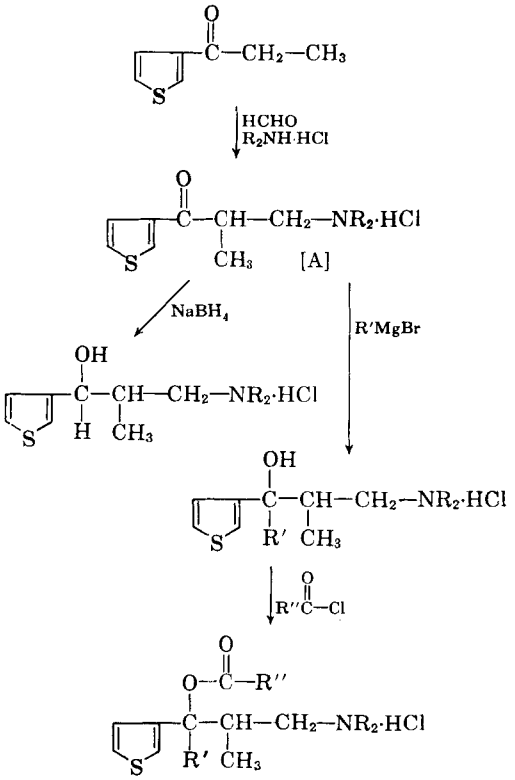
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corresponding secondary alcohol by the use of sodium borohydride. A Type III compound was synthesized by means of refluxing the sodium salt of the alcohol with propionyl chloride in dioxane.

The general synthetic scheme utilized in the preparation of these agents follows

GENERAL SCHEME OF SYNTHESIS



Preliminary information (8) indicates that one of the compounds, 3-dimethylamino-2-methyl-1(3-thienyl)-1-phenylpropanol hydrochloride, combined sedative and stimulating properties in a most interesting manner.

EXPERIMENTAL

Type I Compounds

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-propanone Hydrochloride.—By the usual procedure for the preparation of a Mannich base from a ketone, 13.8 Gm. (0.1 mole) of 3-propionylthiophene, 3 Gm. of paraformaldehyde, and 8.1 Gm. (0.1 mole) of dimethylamine hydrochloride were refluxed in 25 ml. of ethanol. After refluxing for 29 hours, the solution was filtered while hot; it was then cooled. Subsequently, a mixture of 15 ml. of acetone and 85 ml. of dry ether was added. The solid obtained was removed by filtration and recrystallized to analytical purity from a solution of ethanol-ether to produce a yield of 9.1 Gm. (42%) of a compound, m.p. 158–160°.

Anal.—Calcd. for $C_{10}H_{16}ClNOS$: C, 51.40; H, 6.90; S, 13.72. Found: C, 51.37; H, 6.96; S, 13.86.

3-(3-Dimethylamino-2-methyl-propanoyl) thiophene.—This free base was obtained by making an aqueous solution of the above hydrochloride alkaline with ammonium hydroxide and ether extracting the resulting product. The ether extract was dried over Drierite and distilled in the range of 104–105°/0.3 mm.

Anal.—Calcd. for $C_{10}H_{15}NOS$: C, 60.85; H, 7.66; S, 16.02. Found: C, 60.73; H, 7.52; S, 16.13.

Type II Compounds

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-propanol Hydrochloride.—Four hundred milligrams of sodium borohydride was added to 50 mg. of 50% methanol at 18–20°. Two and three-tenths grams (0.01 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene hydrochloride in 50% methanol was added dropwise at 20–22°. After the addition, the reaction mixture was warmed to 30°, maintained at this temperature for 30 minutes, and then heated to 50° for 15 minutes. Methanol was removed *in vacuo*. The residue was made basic with 6 *N* sodium hydroxide, extracted with ether, and dried over Drierite. Anhydrous hydrogen chloride was bubbled in. Recrystallization from an ethanol-ether mixture yielded 1.5 Gm. (60%) of a compound which melted in the range of 154–157°.

Anal.—Calcd. for $C_{10}H_{18}ClNOS$: C, 51.10; H, 7.72. Found: C, 51.33; H, 7.83; IR: OH, 303 μ.

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-phenyl-propanol Hydrochloride.—Phenylmagnesium bromide was prepared from 3.16 Gm. (0.2 mole) of bromobenzene, 730 mg. (0.03 mole) of magnesium turnings, and anhydrous ether. The Grignard solution was stirred at room temperature during the dropwise addition of 2.95 Gm. (0.015 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene in 25 ml. of ether. The reaction mixture was stirred and refluxed for 1 hour; the mixture was then decomposed by the addition of 7 ml. of water. The ether layer was separated, the reaction mixture was extracted with two 25-ml. portions of ether, and the ether layers were combined and dried over Drierite. The hydrochloride was prepared in ether solution, using anhydrous hydrogen chloride. Recrystallization from an ethanol-ether mixture yielded 3.7 Gm. (81%) of a compound which melted in the range of 210–212°.

Anal.—Calcd. for $C_{16}H_{22}ClNOS$: C, 61.80; H, 7.13. Found: C, 61.79; H, 7.06.

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1- cyclohexyl - 1 - propanol Hydrochloride.—Cyclohexylmagnesium bromide was prepared from 3.2 Gm. (0.02 mole) of bromocyclohexane, 730 mg. (0.03 mole) of magnesium turnings, and anhydrous ether. The Grignard solution was stirred at room temperature during the dropwise addition of 2.95 Gm. (0.015 mole) of 3-(3-dimethylamino-2-methylpropanoyl)thiophene in 25 ml. of dry ether. The reaction mixture was stirred and refluxed for 1 hour and then decomposed by the addition of 7 ml. of water. The ether layer was separated, the reaction mixture was extracted with two 25-ml. portions of ether, and the ether layers were combined and dried over Drierite. The hydrochloride was prepared in ether solution, using anhydrous hydrogen chloride. A yield of 3.6 Gm. (76%) of the compound which melted in the range of 233–234° was obtained after recrystallization from an ethanol-ether mixture.

Anal.—Calcd. for $C_{16}H_{28}ClNOS$: C, 60.57; H, 8.80; N, 4.39. Found: C, 60.46; H, 8.72; N, 4.10. U.V. max. 233 mμ (5110); 238 mμ shed (5900); 245 mμ shed (3020).

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-benzyl-1-propanol Hydrochloride.—Benzylmagnesium bromide was prepared from 3.4 Gm. (0.02 mole) of benzyl bromide, 730 mg. (0.03 mole) of magnesium turnings, and anhydrous ether. The Grignard solution was stirred at room temperature during the dropwise addit on of 2.9 Gm. (0.015 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene in 25 ml. of dry ether. The reaction mixture was stirred and refluxed for 1 hour; it was then decom posed by the addition of 7 ml. of water. The ether layer was separated, the reaction mixture was extracted with two 25-ml. portions of ether, and the ether layers were combined and dried over Drierite. The hydrochloride was prepared as indicated above. After recrystallization from an ethanol-ether mixture, a yield of 3.8 Gm. (78%) of a compound which melted at 207° was obtained.

Anal.—Calcd. for $C_{17}H_{24}ClNOS$: C, 62.46; H, 7.40; N, 4.29. Found: C, 62.12; H, 7.19; N, 4.38.

3 - Dimethylamino - 1 - propyl - 1 - (3 - thienyl)-2-methyl-1-propanol Methiodide.—Three hundred and ninety-four milligrams (0.002 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene was treated with an equal molar quantity of propylmagnesium bromide in 15 ml. of dry ether; this mixture was refluxed for 2 hours and then decomposed with the addition of water. The ether .ayer was separated and extracted with two 10-ml. portions of ether. The ether layers were combined, dried over Drierite, and then evaporated to dryness. The residue was dissolved in 10 ml. of acetone. The quaternary salt of the free base was formed by the addition of 1 ml. of methyl iodide; 5 ml. of ether was added to precipitate the salt. A yield of 5.2 Gm. (68%) of the methiodide, m.p. 120–127°, was thus obtained.

Anal.—Calcd. for $C_{14}H_{26}INOS$: C, 43.87; H, 6.84. Found: C, 43.38; H, 6.56.

Type III Compound

4 - Dimethylamino - 1 - phenyl - 2 - (3 - thienyl)-3-methyl-2-propanoyloxybutane Hydrochloride.—To 20 ml. of dry dioxane, 46 mg. (0.002 mole) of sodium metal was added, and the mixture was stirred and heated at reflux temperature until sodium sand resulted. Five hundred and eighty milligrams (0.002 mole) of 3-dimethylamino-2-methyl-1-(3-thienyl)-1-benzyl-1-propanol was added slowly and refluxed for 6 hours. The solution was cooled, and 186 mg. (0.0022 mole) of propionyl chloride was added. After refluxing for 6 hours, the mixture was cooled, water was added, and the mixture was made basic to litmus with sodium carbonate. The solution was extracted three times with 20-ml. portions of ether; the combined extracts were dried over anhydrous potassium carbonate, and the compound was converted in the usual manner to the hydrochloride. A yield of 297 mg. (39.4%) of a compound, m.p. 217°, was thus obtained.

Anal.—Calcd. for $C_{20}H_{28}ClNO_2S$: C, 62.89; H, 7.39; N, 3.67. Found: C, 62.61; H, 7.33; N, 3.73.

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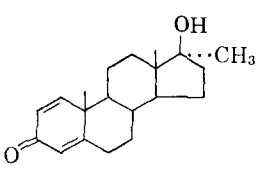
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(8) Preliminary information regarding the nature of the biological activity of this compound was supplied through the courtesy of Schering Corporation, Bloomfield, N. J.

Quantitative Fluorometric Determination of Methandrostenolone

By F. TISHLER, P. B. SHETH, M. B. GIAIMO, and W. J. MADER

A fluorometric procedure is described for the determination of methandrostenolone (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one). The method is based on the flluorogen formed when the steroid is heated with hydrochloric acid at 100°. In order to determine the selectivity of this reaction, a number of related steroids has been studied.

METHANDROSTENOLONE[1] is a new tissue-building compound prepared in these laboratories. Its structural formula is illustrated:



Received March 22, 1962, from the Research Department, Ciba Pharmaceutical Co., Summit, N. J.
Accepted for publication April 11, 1962.
[1] Ciba's trade name for methandrostenolone is Dianabol.

Anal.—Calcd. for $C_{16}H_{28}ClNOS$: C, 60.57; H, 8.80; N, 4.39. Found: C, 60.46; H, 8.72; N, 4.10. U.V. max. 233 mμ (5110); 238 mμ shed (5900); 245 mμ shed (3020).

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-benzyl-1-propanol Hydrochloride.—Benzylmagnesium bromide was prepared from 3.4 Gm. (0.02 mole) of benzyl bromide, 730 mg. (0.03 mole) of magnesium turnings, and anhydrous ether. The Grignard solution was stirred at room temperature during the dropwise addit on of 2.9 Gm. (0.015 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene in 25 ml. of dry ether. The reaction mixture was stirred and refluxed for 1 hour; it was then decom posed by the addition of 7 ml. of water. The ether layer was separated, the reaction mixture was extracted with two 25-ml. portions of ether, and the ether layers were combined and dried over Drierite. The hydrochloride was prepared as indicated above. After recrystallization from an ethanol-ether mixture, a yield of 3.8 Gm. (78%) of a compound which melted at 207° was obtained.

Anal.—Calcd. for $C_{17}H_{24}ClNOS$: C, 62.46; H, 7.40; N, 4.29. Found: C, 62.12; H, 7.19; N, 4.38.

3 - Dimethylamino - 1 - propyl - 1 - (3 - thienyl)-2-methyl-1-propanol Methiodide.—Three hundred and ninety-four milligrams (0.002 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene was treated with an equal molar quantity of propylmagnesium bromide in 15 ml. of dry ether; this mixture was refluxed for 2 hours and then decomposed with the addition of water. The ether .ayer was separated and extracted with two 10-ml. portions of ether. The ether layers were combined, dried over Drierite, and then evaporated to dryness. The residue was dissolved in 10 ml. of acetone. The quaternary salt of the free base was formed by the addition of 1 ml. of methyl iodide; 5 ml. of ether was added to precipitate the salt. A yield of 5.2 Gm. (68%) of the methiodide, m.p. 120–127°, was thus obtained.

Anal.—Calcd. for $C_{14}H_{26}INOS$: C, 43.87; H, 6.84. Found: C, 43.38; H, 6.56.

Type III Compound

4 - Dimethylamino - 1 - phenyl - 2 - (3 - thienyl)-3-methyl-2-propanoyloxybutane Hydrochloride.—To 20 ml. of dry dioxane, 46 mg. (0.002 mole) of sodium metal was added, and the mixture was stirred and heated at reflux temperature until sodium sand resulted. Five hundred and eighty milligrams (0.002 mole) of 3-dimethylamino-2-methyl-1-(3-thienyl)-1-benzyl-1-propanol was added slowly and refluxed for 6 hours. The solution was cooled, and 186 mg. (0.0022 mole) of propionyl chloride was added. After refluxing for 6 hours, the mixture was cooled, water was added, and the mixture was made basic to litmus with sodium carbonate. The solution was extracted three times with 20-ml. portions of ether; the combined extracts were dried over anhydrous potassium carbonate, and the compound was converted in the usual manner to the hydrochloride. A yield of 297 mg. (39.4%) of a compound, m.p. 217°, was thus obtained.

Anal.—Calcd. for $C_{20}H_{28}ClNO_2S$: C, 62.89; H, 7.39; N, 3.67. Found: C, 62.61; H, 7.33; N, 3.73.

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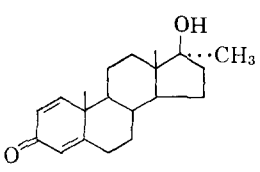
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Quantitative Fluorometric Determination of Methandrostenolone

By F. TISHLER, P. B. SHETH, M. B. GIAIMO, and W. J. MADER

A fluorometric procedure is described for the determination of methandrostenolone (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one). The method is based on the flluorogen formed when the steroid is heated with hydrochloric acid at 100°. In order to determine the selectivity of this reaction, a number of related steroids has been studied.

METHANDROSTENOLONE[1] is a new tissue-building compound prepared in these laboratories. Its structural formula is illustrated:



Received March 22, 1962, from the Research Department, Ciba Pharmaceutical Co., Summit, N. J.
Accepted for publication April 11, 1962.
[1] Ciba's trade name for methandrostenolone is Dianabol.

The ultraviolet spectrum of methandrostenolone in methanol appears in Fig. 1 and can be used for the quantitative determination of the compound; however, ultraviolet analysis in methanol cannot be employed when methyltestosterone is present as an impurity since each steroid exhibits a maximum around 241 mμ.

Fluorometry offers a means to the qualitative and quantitative determination of microgram amounts of steroids. Sulfuric acid has been employed by many workers (1–4) to form the fluorogen used for the determination of various steroids. Recently Touchstone and Murawec (5) found that certain steroids exhibited a greater fluorescent intensity when heated with a 2*N* methanolic potassium hydroxide solution prior to being dissolved in sulfuric acid than when dissolved in sulfuric acid alone. The use of hydrochloric acid to induce fluorescence in steroids has also been reported (6–8).

It has been observed in these laboratories that when methandrostenolone is heated at 100° with hydrochloric acid a fluorogen is formed, which is the basis of the method of analysis to be described. Under the same conditions, methyltestosterone exhibits no fluorescence, thereby offering a means of determining methandrostenolone in which methyltestosterone might be present as an impurity. Optimum conditions for the hydrochloric acid-induced fluorescence have been determined and a number of related steroids have been studied in order to determine the selectivity of this reaction.

EXPERIMENTAL

Apparatus.—All measurements are made on an Aminco-Bowman spectrophotofluorometer with a quartz cell having a 1-cm. light path using a $^1/_{16}$-in. defining slit (band pass = 6 mμ) and an RCA 1-P-21 photomultiplier. The excitation and fluorescent spectra are obtained on an Electro model 101 XY recorder. The instrument is calibrated against a quinine solution by the method of Sprince and Rowley (9). Absorbance measurements are made with a Cary recording spectrophotometer.

Reagents.—All steroids used in the study are of reference standard quality, either assayed by phase solubility (10) or purchased from the U.S.P. as reference standards. All other reagents are reagent grade. Standard methandrostenolone solutions are prepared containing 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 mcg./ml. in methanol.

Procedure.—Weigh a counted number of not less than 20 tablets and reduce them to a fine powder. Transfer an accurately weighed portion of the powder, equivalent to 10 mg. of methandrostenolone, to a 100-ml. volumetric flask. Add 75 ml. of methanol, shake for 10 minutes, dilute to volume with methanol, and mix well. Centrifuge a portion of the mixture. Transfer a 5.0-ml. aliquot sample of the clear solution to a 100-ml. volumetric flask, dilute to volume with methanol, and mix well. (Concentration = 5.0 mcg./ml.)

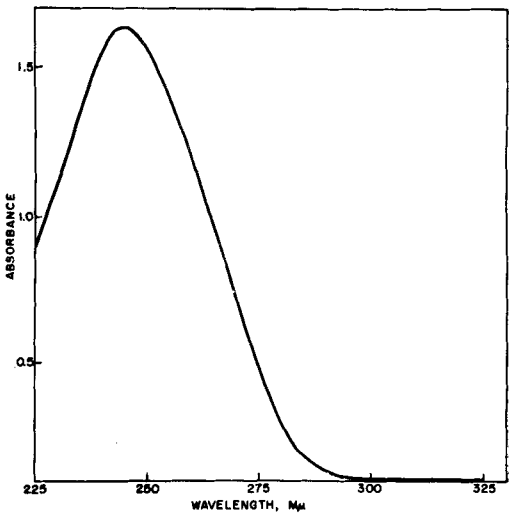


Fig. 1.—Ultraviolet absorption spectrum of methandrostenolone in methanol, 0.01 mg./ml.

Pipet 2.0 ml. of the sample preparation, 2.0 ml. of a standard solution (5.0 mcg./ml.), and 2.0 ml. of methanol into separate 25-ml. volumetric flasks. Add 5 ml. of concentrated hydrochloric acid and heat in an oven at 100°. After 20 minutes, wash down the sides of the volumetric flasks with 2 ml. of methanol and continue heating for an additional 20 minutes. Cool the flasks to room temperature, dilute to volume with methanol, and mix well. Read the fluorescent intensity of the blank, sample, and standard solutions at an excitation of 280 mμ and a fluorescence of 345 mμ after setting the photometer sensitivity to full and the meter multiplier to 0.03. Calculate the amount of methandrostenolone in each tablet by the following formula

$$\frac{A_o - A_b}{A_s - A_b} \times 10 \times \frac{Wt}{Ws} =$$

mg. of methandrostenolone/tablet

where Ao = fluorescent intensity of the sample, As = fluorescent intensity of the standard, Ab = fluorescent intensity of the blank, Wt = average weight per tablet in Gm., and Ws = weight of sample in Gm.

DISCUSSION

When exhibited, excitation and fluorescent maxima were obtained for all steroids used in this study. The excitation and fluorescent spectra for methandrostenolone appear in Fig. 2. In order to determine the optimum conditions for the formation of the fluorogen, the fluorescence of the final solution as a function of time and milliliters of hydrochloric acid added was investigated. From Figs. 3 and 4 it can be seen that at 100°, a constant maximum fluorescence is obtained after 20 minutes using 4 ml. of hydrochloric acid. To be sure of maximum fluorescent intensity, 5 ml. of acid was used in the procedure. It was found that, due to evaporation of the solvent over the required heating period, reproducible results were not always obtained be-

cause of unreacted methandrostenolone on the sides of the flask. For this reason, a second heating period was employed after washing the flasks with 2 ml. of methanol. A plot of fluorescent intensity *vs.* concentration was found to be linear up to 30 mcg./25 ml. of final solution (Fig. 5).

Temperatures below 100° were unsatisfactory, and at room temperature the fluorogen was not formed. Dilution with water or dilute hydrochloric acid rather than methanol decreased the fluorescent intensity in all cases.

As a test of the applicability of the fluorometric procedure to pharmaceutical preparations, several

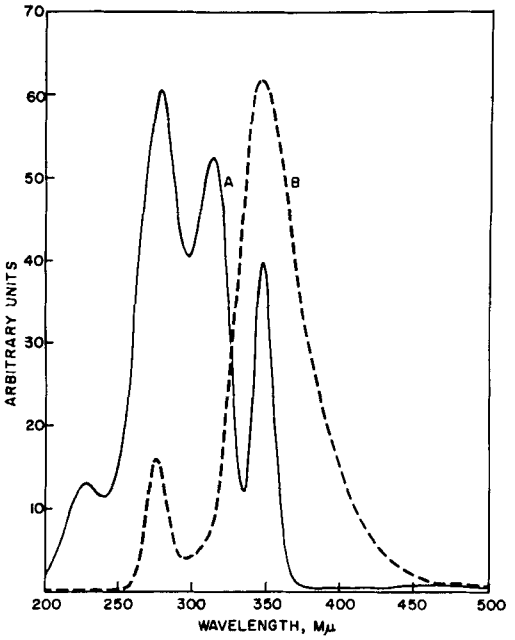


Fig. 2.—Excitation and fluorescent spectra of methandrostenolone. *A*, Excitation scan, fluorescence held at 345 mμ; *B*, fluorescent scan, excitation held at 280 mμ.

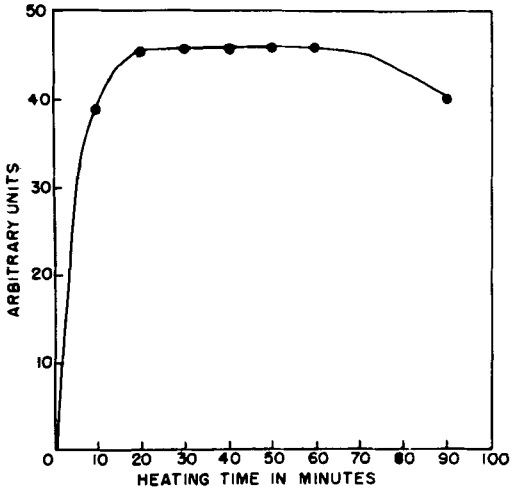


Fig. 3.—Plot of fluorescences of final solution against heating time of reaction.

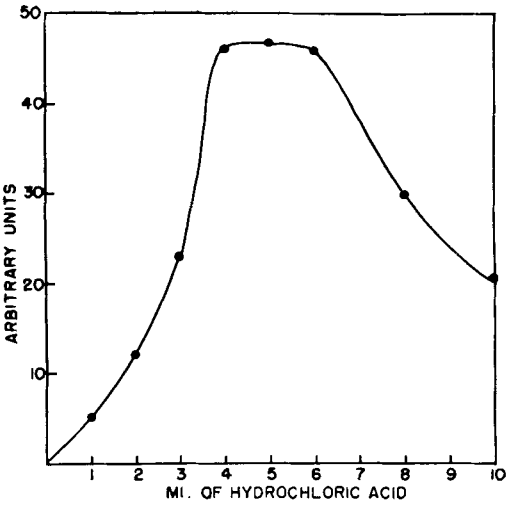


Fig. 4.—Plot of fluorescences of final solution against milliliters of concentrated hydrochloric acid.

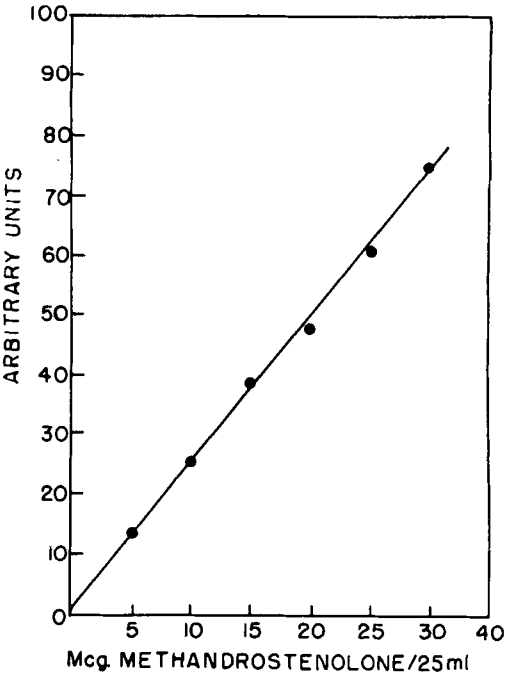


Fig. 5.—Plot of fluorescences of final solution against concentration of methandrostenolone (0.1 meter multiplier).

lots of tablets containing methandrostenolone were analyzed by both the proposed method and by ultraviolet analysis. The results appear in Table I. The results on several commercial lots, performed in triplicate, showed a precision of ±2%.

Common tablet excipients did not interfere with the fluorescent technique. In order to determine whether methyltestosterone interfered with the fluorometric procedure, several samples of methandrostenolone containing up to 10% of methyltestosterone were assayed for methandrostenolone. Methyltestosterone itself showed no fluorescence

TABLE I.—COMPARISON OF TABLETS BY FLUORESCENT AND ULTRAVIOLET PROCEDURES

Sample	Description and Declaration, mg.	Methandrostenolone Found, mg. Fluorescent	Ultraviolet
1	2.5	2.47	2.49
2	2.5	2.48	2.50
3	5.0	4.94	5.02
4	1.0 with 9α-fluoro-16α-methylprednisolone[b]	1.05	1.04[a]

[a] A preliminary extraction and separation is necessary before U.V. analysis can be employed. [b] Ciba's trade name for 9α-fluoro-16α-methylprednisolone is Gammacorten.

with hydrochloric acid but when present in concentrations greater than 8%, complete development of the fluorogen was inhibited.

A number of related steroids was studied in order to determine the selectivity of the reaction and the effect of various groups on the molecule (see Table II). Of the various steroids studied, only methandrostenolone, ethinyl estradiol, 17α-methylestradiol, and 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one showed fluorescence under the conditions employed. The excitation spectrum which is the same for ethinyl estradiol and 17α-methylestradiol appears in Fig. 6. From Figs. 2 and 6, it can be seen that methandrostenolone can be readily distinguished from ethinyl estradiol and 17α-methylestradiol as the latter compounds show no maximum at 310 mμ; however, methandrostenolone cannot be distinguiushed from 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one as both excitation and fluorescent spectra are very similar.

It is, therefore, indicated that hydrochloric acid-induced fluorescence is applicable only to methandrostenolone and certain structurally related steroids. The reaction appears to be selective for $\Delta^{1,4}$-dien-3-one or $\Delta^{1,3,5,(10)}$-trien-3-ol steroids which have both a 17β-hydroxy and 17α-alkyl or alkyne substitution. Further studies are being carried out to determine the chemistry of the reaction of hydrochloric acid-induced fluorescence and to determine the effect of various other substitutions at the 17α-, 17β-, and 3-positions.

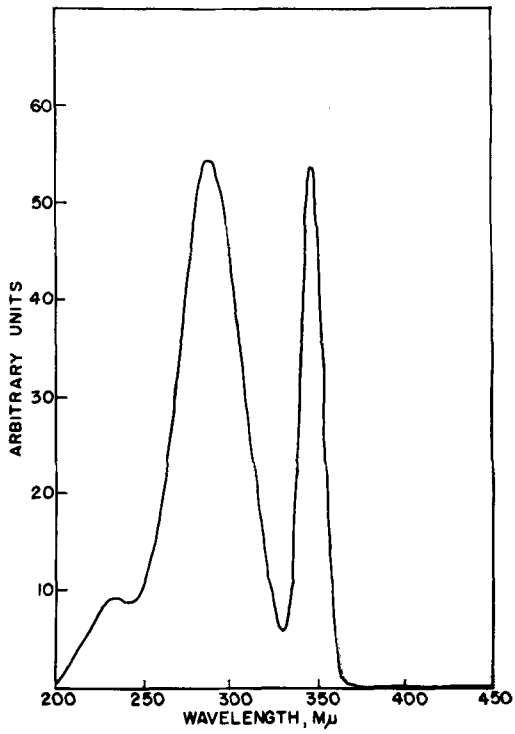


Fig. 6.—Excitation spectrum for both ethinyl estradiol and 17α-methylestradiol, fluorescence held at 345 mμ.

SUMMARY

1. A method is described for the quantitative fluorometric determination of methandrostenolone.

2. A number of related steroids is studied to determine the selectivity of hydrochloric acid-induced fluorescence

TABLE II.—FLUORESCENCE OF 17α-METHYL-17β-HYDROXYANDROSTA-1,4-DIEN-3-ONE AND SOME RELATED COMPOUNDS

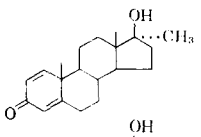
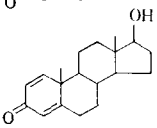
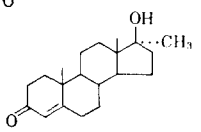
Compound	Structure	Relative Fluorescent Intensity
17α-Methyl-17β-hydroxyandrosta-1,4-dien-3-one		100
17β-Hydroxyandrosta-1,4-dien-3-one		Nil
Methyltestosterone		Nil

TABLE II (*Continued*)

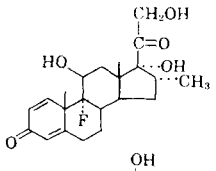
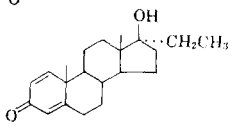
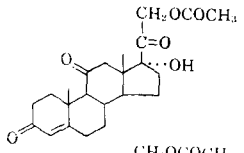
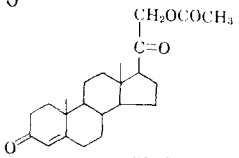
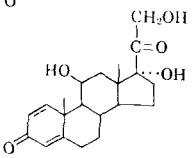
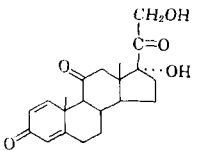
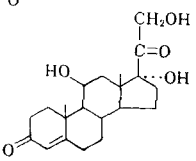
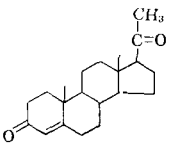
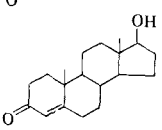
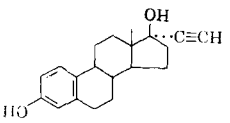
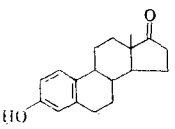
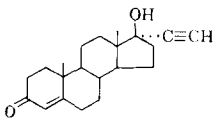
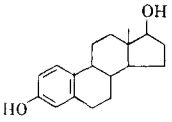
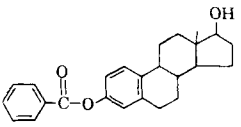
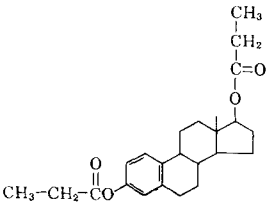
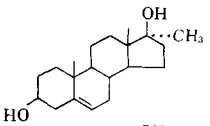
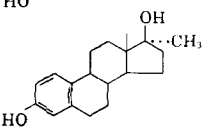
Compound	Structure	Relative Fluorescent Intensity
9α-Fluoro-16α-methylprednisolone		Nil
17α-Ethyl-17β-hydroxyandrosta-1,4-dien-3-one		80
Cortisone acetate		Nil
Desoxycorticosterone acetate		Nil
Prednisolone		Nil
Prednisone		Nil
Hydrocortisone		Nil
Progesterone		Nil
Testosterone		Nil
Ethinyl estradiol[a]		48
Estrone		Nil

TABLE II (*Continued*)

Compound	Structure	Relative Fluorescent Intensity
Ethisterone		Nil
β-Estradiol		4
Estradiol benzoate		Nil
Estradiol dipropionate		Nil
17α-Methyl-5-androstene-3β,17β-diol		11
17α-Methylestradiol[a]		81

[a] A maximum fluorescent reading is obtained at an excitation of 290 mμ and a fluorescence of 345 mμ.

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A Punch and Die Control Program and Its Contribution to Tableting Technology

By C. J. SWARTZ, S. WEINSTEIN†, J. WINDHEUSER‡, and J. COOPER

The necessity for a comprehensive quality control program for tableting tools is discussed and the establishment of tool specifications and inspection facilities are described. Several experiments are presented leading to the evaluation of steels, tool shapes, tolerances, granulations, and tool maintenance on the effective life span of the tableting tools.

IN RECENT years tablets have become the most widely employed mode of administering drugs orally. As a result, the techniques involved in their preparation have been under the intensive scrutiny of pharmaceutical researchers to a continually greater degree. The spirit of criticism and inquiry which has developed in this important area of technology has produced many new innovations in techniques and equipment for tableting operations. The latter developments have included multilayer and compression coating machinery as well as increasingly higher speed presses equipped with induced or forced feeders.

Although these more complex devices have undergone considerable study, the important area of tableting tools has been left relatively untouched by both the tablet machinery manufacturers and the industries which employ them. An awareness of this deficit has recently been referred to in two articles published by the tableting tool industry. Gaskell (1) has stated that "the quality of the finished product depends in no small measure on the quality of the tools which actually compress the powders." It is not difficult to realize that with machines with outputs of 3000 to 6000 tablets/min., many tooling difficulties never previously encountered could be anticipated. Heavy losses in productivity resulting from poor quality punches and concurrent frequent tool replacement certainly must be avoided. If the critical variables cannot be adequately controlled, the utility of the high-speed machinery is seriously hampered. Bobbitt (2) has further noted that "mechanical perfection as well as selection of the proper tooling materials are both necessary precursors to permit modern presses to deliver the precision performance and large volume output that has been engineered into them."

Despite commentary such as the above from punch and die manufacturers indicating the need for exacting and reproducible tool quality, experiences at Ciba disclosed a definite need for the initiation of a routine quality control program for incoming tableting tools. To substantiate this, a few specific examples might be of value.

Aside from the normal processing errors that were encountered in dealing with suppliers, numerous problems have developed of which the following are representative: oversized and undersized punch heads, shank diameter irregularities, oversized punch tips, improper punch face finish including surface polish, monogram irregularities, nonuniformity of steel hardness throughout tool, overall punch length variations, and nonconcentricity of die bore and punch tip.

Prior to the initiation of an inspection program, our concept of the causes of the tooling problems which beset us was limited. However, inspection of incoming punches and dies revealed a vast array of nonconformance to specifications set up by the tool suppliers themselves. Certainly no comment is required relevant to the potential difficulties which would be encountered when using unsatisfactory tooling as described above.

It might be advantageous to dwell in greater depth on the subject of punch face characteristics. An example of an unsatisfactory finish on a punch face can be observed in Fig. 1, the obvious irregularities being readily apparent. It takes little imagination to appreciate what effect a punch finish such as this would have on the tablets compressed from it. The results obtained when these punches were returned to the manufacturer for corrective action can be seen in Fig. 2. The faces have been overbuffed and, although the buffing considerably improved the appearance of

Received February 6, 1962, from the Research Dept., Ciba Pharmaceutical Co., Summit, N. J.
Accepted for publication May 11, 1962.
Presented in part to the First Annual Eastern Regional Meeting of the A.PH.A. Industrial Pharmacy Section, New York, N. Y., November 13, 1961.
The authors gratefully wish to acknowledge the contributions made by Messrs. Walter Steinert and George Kessler of the Ciba Plant Service Division.
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Fig. 1.—Punch tip showing surface irregularities.

the face of the punch, it also removed a significant amount of the embossed monogram, particularly the letter A. These punches were rejected a second time. Unfortunately, the above was not an isolated example of unsatisfactory tools obtained from the punch and die manufacturers.

In the pharmaceutical industry controls are established over all aspects of manufacturing procedures. Since tooling is one of the major factors governing the quality of finished tablets, a program of inspection must be considered to be in the category of other necessary incoming material inspections, such as packages and chemicals. Since machine problems often cause considerable production delays as well as postponement of product introductions, we felt that it was important to initiate a more comprehensive quality control program for our tableting tools. As a result, studies were undertaken to develop a standardized program for tool purchase and control.

DESIGN OF THE CONTROL PROGRAM

The objective of a control program for punches and dies, as in any other quality control program, was to define clearly and precisely the tooling desired and subsequently to maintain this high quality of tooling through careful inspection. The first step toward our objective involved the establishment of tool specifications and tolerances for all punches and dies acceptable both to Ciba and the tool suppliers.

Development of Specifications.—The development of satisfactory tool specifications required the combined efforts of several divisions, including Pharmaceutical Manufacturing, Plant Engineering, Purchasing and Pharmacy Research, as well as the cooperation of the tool manufacturer. The specifications established were based upon the requirements of the machines used, the products being manufactured, and the working tolerances of the tool manufacturers. To obtain maximum flexibility, a system of drawings was developed encompassing monograms, punch blanks, punch tips, dies, and special shaped punch and die sets.

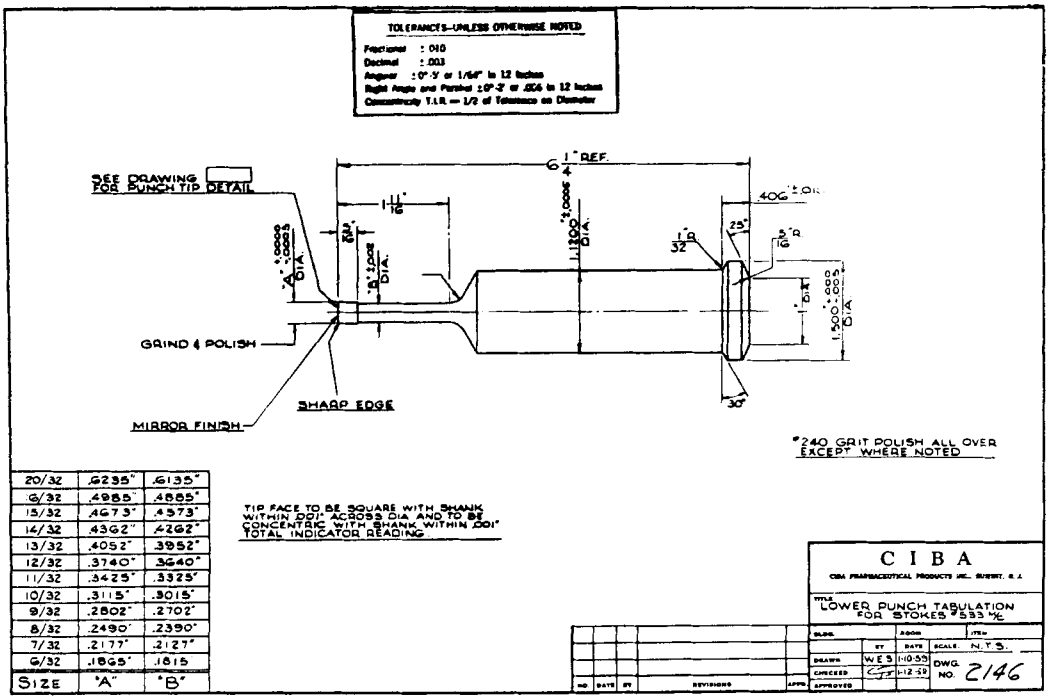
Preliminary drawings of all tools used were developed and eventually assumed the form seen in Fig. 3. This representative example is a typical punch body drawing with carefully evaluated tolerances on all critical dimensions. All specification drawings include tooling size ranges from $^{6}/_{32}$ to $^{20}/_{32}$ inch by the use of appropriate tabulations. Separate punch tip detail drawings define tip shape, depth of concavity, types of monogram, and the length of the tools. In order to reproduce the quality of the punch face it was necessary to control the quality of the hobs being used by the manufacturer. This required the preparation of monogram drawings which defined tolerances for every facet of the monogram, including spacing, depth, and overall length of each of the letters. Further, in consideration of recent tablet counterfeiting attempts, it is evident that the development of such precisely controlled monograms would facilitate the detection of these criminal acts.



Fig. 2.—Punch tip after buffing.

Inspection Program.—The establishment of specifications as described above did not, in itself, solve the problem of tool control. The initiation of a 100% inspection program for incoming punches and dies was found to be necessary. Engineers and mechanics, utilizing appropriate instruments, developed standard techniques for checking all critical dimensions. Figure 4 presents a view of the inspection area showing the equipment used for checking all critical dimensions. Present are measuring tools, including indicating micrometers, gauge blocks, an indicating bore gauge, and a master form gauge. This precision equipment can measure to an accuracy range of ±0.0001 in. The inspector checks the dimensions against the drawings which were sent with the tool order to the manufacturer. Other inspection devices include a depth measuring stereomicroscope to measure punch face characteristics. A steel hardness tester is also available, so that punches and dies may be checked not only for conformance to specifications, but also for uniformity of steel temper throughout each tool. The steels currently used for tooling are based upon the suppliers' recommendations.

If the complete set of punches or dies meets all the standards, they are then accepted and etched



SIZE	"A"	"B"
20/32	.6235"	.6135"
19/32	.5935"	.5835"
18/32	.5635"	.5535"
17/32	.5335"	.5235"
16/32	.5035"	.4935"
15/32	.4735"	.4635"
14/32	.4432"	.4332"
13/32	.4052"	.3952"
12/32	.3740"	.3640"
11/32	.3425"	.3325"
10/32	.3115"	.3015"
9/32	.2802"	.2702"
8/32	.2490"	.2390"
7/32	.2177"	.2127"
6/32	.1865"	.1815"

Fig. 3.—Print showing punch body specifications.

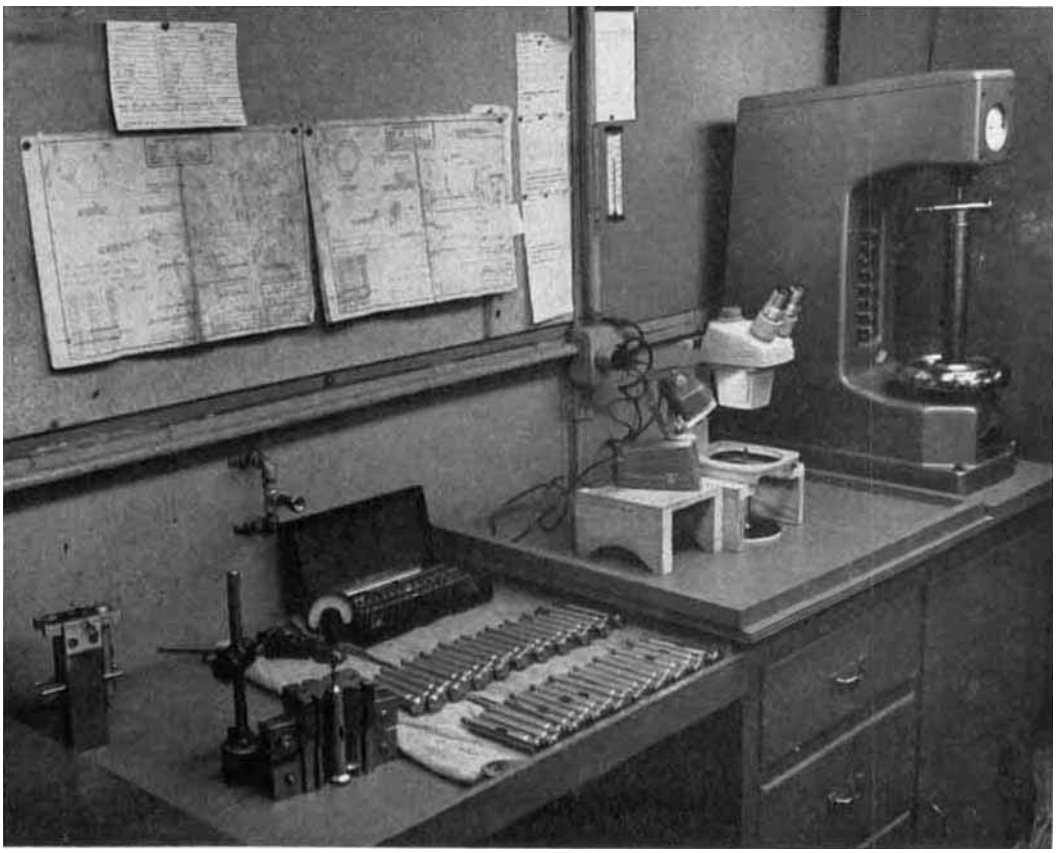


Fig. 4.—Tool inspection area with testing equipment.

with a control number for identification. Should the tools fail to meet specifications, they are rejected and returned to the manufacturer for reprocessing or replacement.

Considering aspects of the inspection procedure, all the dimensions defined are important and are checked. However, through our experience, several areas have been found to be of more critical concern and limits of ±0.0005 in. have been established for them. These regions include head thickness, overall punch length, and shank diameter. Justification for this may best be described by way of example. During compression, the influence of variation in overall length of the lower punches will affect weight variation between tablets, thus altering the dose finally administered to the patient. Hardness of the tablets will not be uniform and the mechanics of compression coating operations may be seriously affected. Tapered and oversized punch shanks can lead to seizing of the punch body in the head of the machine, with resultant severe turret damage. The concentricity of the die bore, in relation to the concentricity of the punch tip, if not within specified tolerances can seriously affect continuous machine operation.

After the first year of the program we were surprised to see that 33.8% of all incoming tooling was rejected as not conforming to specifications. This was particularly disturbing since these standards were set up in conjunction with our tooling suppliers, using their working tolerances. Since then, as can be noted in Fig. 5, we have experienced a steady reduction in the total number of tools returned to the fabricators. It is felt that a paramount factor in this reduction has been the establishment and maintenance of our vigorous inspection program. The continued progress achieved in this area is indicated by the further reduction of rejections to a level of 4.0% of all tooling received during 1961. While the decrease in the total number of rejections during the fourth year of the program is heartening and demonstrates positive action on the part of our suppliers, the continuing appreciable number of unsatisfactory punches and dies points to the need for maintenance of total inspection as it is presently being carried out.

Recording Tool Life History.—The establishment of specifications and subsequent quality control of the tools will insure a uniform high quality in the tools purchased. But, at our present level of knowledge in this area, we cannot be certain that the quality of tooling now used, however uniform, is the most satisfactory for uninterrupted production. So that we might obtain such information, a system for recording the life of the various tools has been developed which enables us to know the total number of tablets compressed with any given set of tools. In addition, information on the products prepared, machines the tools were used with, and the reason that the tools were eventually discarded is recorded. The careful evaluation of the facts obtained from these records will be useful in purchasing tooling with optimum wear characteristics and will result in a better finished product at reduced cost.

The tool life information is recorded on specially prepared cards. They may disclose the merits of refacing procedures, in those instances where refacing is feasible. The records will also show whether misuse by operators, excessive wear by the machine, or a particular granulation was the principal reason that the tools are no longer suitable for use. Since, for the purpose of accurate wear records it is necessary to employ tools only as complete sets, the record cards give the extra benefit of an auditing control of each set of punches for inventory purposes.

RESEARCH AND DEVELOPMENT ASPECTS

Over and above the advantages derived from a quality control viewpoint, the tableting tool control program as just outlined has been responsible for a number of benefits, of both a general and specific character, in tablet manufacturing at Ciba. The acquisition of tooling of desired specifications can now be assured regardless of the source of supply. From the point of view of research and development, the secondary benefits of the program allow the planning and execution of experiments leading to the evaluation of the effects of steels, tool shapes, tolerances, granulation, and tool maintenance on the effective life span of the tableting tools.

Although the program is of recent origin, a number of interesting projects are being pursued and will be discussed below.

Effect of Steel on Tool Life.—From intuitive and cursory evaluation of steels based on data gathered prior to the introduction of the punch and die program, it appeared evident that the type and hardness of steel used in the punches had a profound effect on the life of the tools being used. To accelerate the development of more basic scientific knowledge in this area, an experiment was devised in which a set of punches was prepared from four different types of steels, each being tempered to the same hardness. The test conditions were kept constant by the utilization of a 35-station tableting press and a single product, the only variable being the steel employed for the punches.

Although this study is still in progress, preliminary examination of punches by photomicrography indicates that there are definite differences in the wear qualities of each of the steels used, even though all have the same hardness. Upon completion of this study, or ones of similar design, one will be able to arrive at a scientific conclusion as to the relative merits of the various steels, based upon experimental data rather than speculation. It must be

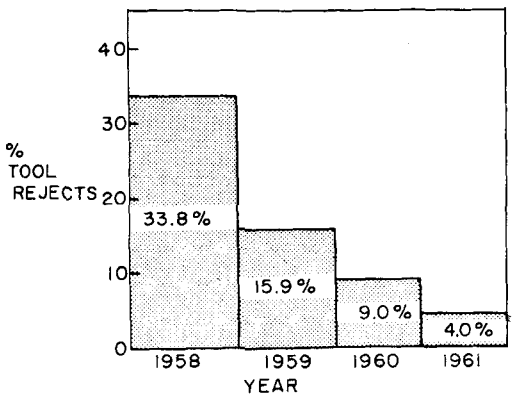


Fig. 5.—Rejection rate of tooling following the introduction of the control program.

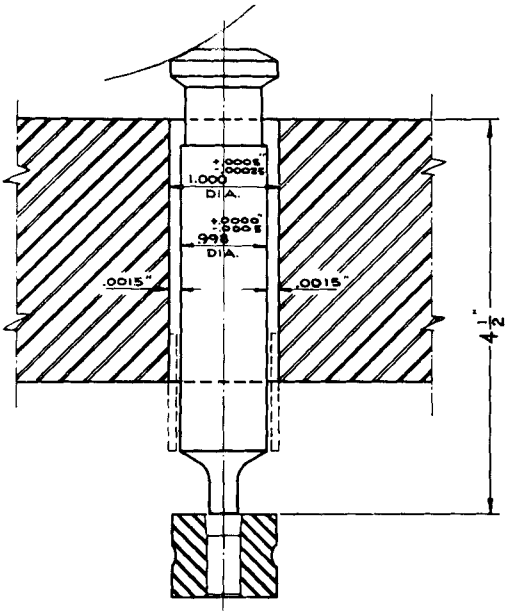


Fig. 6.—Cross-section of upper punch in guide bore with established specifications.

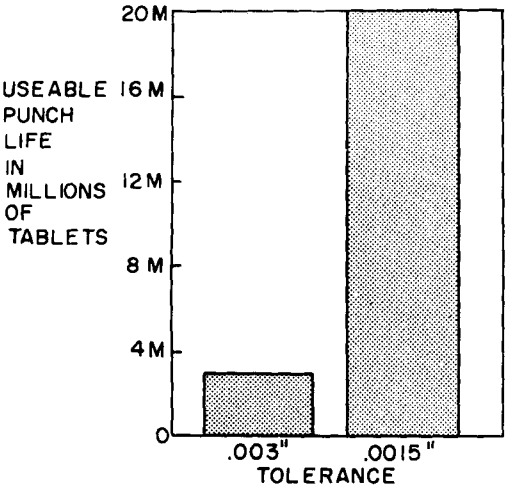


Fig. 7.—Added tool life attributed to closer tolerances.

emphasized that these results can only be accepted as valid in respect to the product tested as it has been shown that different formulations of the same product may possibly alter punch life characteristics.

Effect of Punch Shape.—As was noted earlier, evaluation of the records obtained to date indicated that the shape or geometry of punch tips had a marked influence on the life of punches. The following shapes commonly employed in the manufacture of pharmaceutical tablets are listed in an order of the most durable to the least durable shape: flat, shallow concave, standard concave, flat beveled, deep concave, modified ball punches.

The reason for the order, which has been established from examination of our records, can be explained on the basis of edge strength. It has been found, upon study of unsatisfactory punches, that a common reason for discarding them is the development of a rolled-in burr. It is readily apparent that a modified ball punch tip which, by design, has extremely sharp and narrow edges would be much more prone to this type of deformation than the flat punch, all other shapes fitting somewhere in between. It has been found that the shape of punch tips is also important with respect to misalignment or poor fit in the tableting press itself. This will be discussed in the next section.

Effects of Machine Tolerances.—During the evaluation of data it was noted that, in some cases, a marked decrease in the expected life of the tools resulted when tools were used on certain machines. It was found in all instances that the reduction of tool life was due to damage to the upper punches in the form of a rolled-in lip or burr. Stroboscopic examination of the machine in operation at production speeds indicated that enough clearance existed between the punch shank and machine bore to allow considerable "run out" of the tool at the die table during the compression cycle. This "run-out" results from the eccentric force exerted by the upper pressure roller which forced the tool out of line to as much as 0.010 inch off center at the point of entry into the die. This constant contact with the die edge very rapidly resulted in the lapped condition observed. The solution to this dilemma was a cooperative effort between ourselves and the manufacturer who prepared a new head for the machine in question utilizing tighter clearances for the upper punch, as noted in Fig. 6. Calculations indicated that reducing the clearances between the punch shank and the bore to 0.0015 in. from the 0.003 in. normally allowed on the machine would be the most suitable solution to the problem. Following the receipt of a head, machined to the dimensions specified, experiments were conducted to determine the influence of the turret with these closer tolerances on the actual wear characteristics of the punches in question. As evidenced in Fig. 7 there is no question about the added tool life which can be attributed to closer tolerances. The graph clearly illustrates that, to date, we have obtained at least six times greater usable life from the punches by the application of closer tolerances. It is readily apparent that although the original program was not intended to study the compression machines, *per se*, the availability of accurate, recorded data on punch life enabled us to become aware of a machine problem and to facilitate its solution readily. The economic advantages of a scientific approach to tableting are obvious in this instance.

Effect of Granulations.—Up to this point we have dwelled mainly upon the mechanical aspects of the program, but any tableting operation can be considered a three-component system consisting of the machine, the tools, and the granulation. All things being equal, the active agent and/or excipients of the granulations exert a profound effect on the life of the tools being used. Again, from experiences prior to the initiation of this program, it was our impression that the marked variations in the length of tool life were directly related to the products compressed. Examination of the records of discarded sets having been controlled and used under the conditions of the program indicated our conjec-

ture to be well founded. This can be most dramatically illustrated by the following: glutethimide (Doriden, Ciba) tablets are prepared utilizing $^{15}/_{32}$ in. flat beveled punches. Records indicate that, under the conditions of manufacture and using what will be called formula A, a yield of approximately 25,000,000 tablets per set of punches could be expected on a 35-station machine. For other technical reasons it became necessary to modify the formulation to formula B. Concurrently with this, a new set of punches meeting the same specifications as those previously used were ordered and used exclusively with formula B. The completed report on these tools indicates that 114,000,000 tablets have been compressed on the same machine under the same conditions. This 4.5-fold increase in the life of the tools is a significant change and can be wholly attributed to modification of the granulation components. Although a replacement set is still being used, a check at this date indicates that 80,000,000 tablets have been produced so far from this second set without deleterious effects on the tools. This would suggest that the initial results with the new formulation will be substantiated by the second set of punches now in use.

Relationship of Dies to the Life of Punches.—Our consideration thus far has been mainly devoted to the life of the punch due to the relative costs involved. Inasmuch as one would be prone to examine punches more thoroughly than dies, it should be pointed out that in the majority of our trials, carbide lined dies have been utilized. No data are available as yet on the length of service of controlled sets of these dies as the carbide lining appears impervious to normal wear, with the exception of damage brought about by sudden shocks. It has been noted that in the cases of modified ball, deep concave, and flat beveled punch tips, carbide dies, acting as honing surfaces, have a tendency to wear the edges of the punches rapidly.

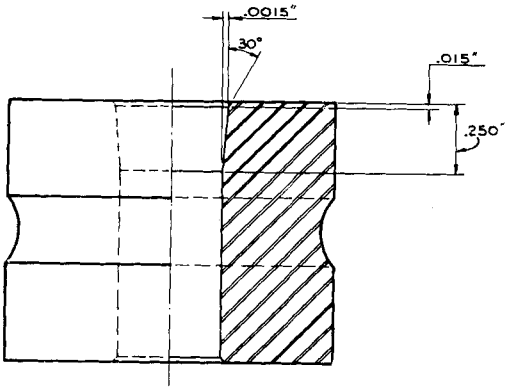


Fig. 8.—Die with tapered bore.

An interesting facet examined is the use of tapered dies. In these dies the bore is not a uniform cylinder but gradually tapers, as shown in Fig. 8, or is gradually tapered completely through the bore. It has been suggested that the tapering would reduce die wall friction during the ejection cycle by virtue of the increase in die diameter anywhere above the compression point. It was felt that if this were true it could be demonstrated by differences between edge

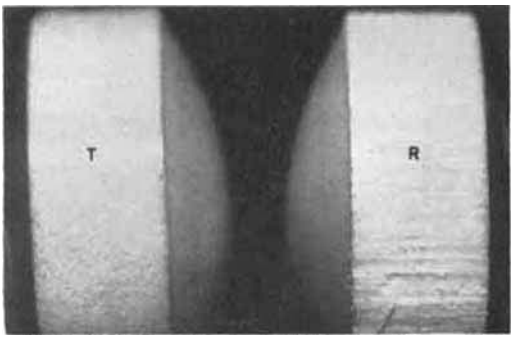


Fig. 9.—Tablets prepared using tapered (T) and regular (R) dies showing edge characteristics.

characteristics of tablets compressed in standard or tapered dies. Figure 9 shows the results of an experiment carried out at Ciba comparing tapered and standard dies. The test substance consisted of granulated magnesium oxide (granulated with sucrose solution) and stearic acid *q.s.* The amount of stearic acid was reduced successively until binding occurred in standard bore dies. At this point, tapered dies were substituted and the same granulation compressed. The different edge characteristics noted upon examination of Fig. 9 showing the regular signified by "R" and the "T" signifying tapering dies, clearly indicated that tapered dies prevented binding. It is fully recognized that this is a qualitative rather than a quantitative analysis, but it does indicate the possible value of the substitution of tapered dies in selected problem products. The reduction of binding by the tapered dies allows the use of lower concentrations of lubricants which, as shown by Strickland, *et al.* (3), generally results in lowering of disintegration time of tablets.

CONCLUSION

The comprehensive integration of pharmaceutical requirements and tool technology under the program has been found to be a valuable stimulus to investigation of tableting problems. It is impossible to contemplate research projects in tableting without a thorough understanding of the influence exerted by the tooling on the resultant data. It is obvious that marked variations of the tooling from established specifications could invalidate conclusions drawn from otherwise closely controlled experiments.

For years pharmaceutical manufacturers have accepted the expense of nonuniform punches and dies, fearing that a program to improve their caliber would be even more costly. It has been found that the ability to introduce new products on schedule, elimination of severe machine damage, and prolonging of tool life more than compensates for the costs involved. Aside from the technical and financial aspects, there is no question that well prepared tooling helps insure uniformity of finished tablets which is, after all, the paramount aim of our science.

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Acceptance Sampling of Finished Pharmaceutical Products

By H. LATHAM BREUNIG and E. P. KING

In this paper we describe some problems inherent in acceptance sampling of finished pharmaceuticals, and provide an expository account of the power and effectiveness of modern statistical methods in the solution of these problems.

STATISTICAL techniques form an integral part of every effective quality control program. Sound statistical procedures for accepting or rejecting batches of finished product based on evidence obtained from samples were given tremendous impetus during World War II through their recognition and application by military procurement agencies. Since that time, these methods have enjoyed a continually expanding circle of usage in the chemical and process industries.

On the other hand, criteria currently used for acceptance sampling of medicinal products, based upon chemical or biological assay, are susceptible to considerable improvement. It would further appear that as this need for improvement becomes recognized and as changes in the sampling procedures are proposed, even these revisions do not incorporate the efficient statistical methods now available.

The sampling procedures of today are probably the result of a natural process of evolution. For many years the majority of active drugs were dispensed in the form of fluid extracts and solutions. The first official sampling procedure to appear in the United States Pharmacopeia (1) pertained to the assay of crude drugs which were to be extracted. Because the liquid extract was recognized as homogeneous, it was natural that attention be shifted from the finished product and focused on accurately estimating the amount of active principle in a shipment of crude drug. It was logical to strive for a "representative sample" of the shipment, ignoring variation from portion to portion within a shipment, because the entire shipment would ultimately be extracted as one unit to form a homogeneous fluid. Hence the procedure called for taking core samples, compositing, quartering, and the like.

However, as time passed, new dosage forms came into prominence. Tablets and filled capsules replaced fluid extracts and solutions. Since this was a gradual process, the sampling procedures for crude drugs were carried over unchanged to the new forms. Unfortunately, however, the problem had changed. Unit-to-unit variation among finished tablets or capsules from the same batch is inherently greater than the variation from one portion of a fluid to another. A thoroughly mixed fluid is homogeneous throughout, but two tablets from the same batch can differ in potency because of composition variation in granulation and because of weight variation among the tablets after compression.

Both physician and patient are concerned with whether or not the amount of drug in a single dose conforms to label claim. Estimating the average amount of drug per unit weight in the parent batch is only a first step. The older sampling methods, aimed at assessing the amount of active ingredient in a crude drug shipment, simply cannot be depended upon to provide information about unit-to-unit differences in tablets and filled capsules. Hence the "representative sample" approach, although adequate for its original purpose, must now make room for techniques which concentrate on individual units of finished product; methods which are concerned not only with average drug content but also with variation in potency from unit to unit. Such a program is given further impetus by the development, in recent years, of analytical methods which permit

Received March 22, 1962, from Eli Lilly and Company, Indianapolis 6, Ind.
Accepted for publication May 10, 1962.
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accurate assay for the relatively small amount of active ingredient in each unit.

The need for this type of change has been recognized by many responsible individuals—both within the Food and Drug Administration and within the pharmaceutical industry. Reports such as that by Pernarowski and co-workers (2) have discussed dosage variation among single tablets of several items. However, some of the proposals which have been made still fall far short of the effectiveness that could be achieved through the use of statistical methods of acceptance sampling. Furthermore, reference to sampling plans in *Interim Federal Standard No. 00140 (Navy-Bu Med)* (3) concerning procurement of tablets for medicinal purposes underlines the need for an understanding of the properties of such plans. Our purpose in the following expository discussion is to illustrate the advantages to be had through the application of these modern statistical principles.

Inherent Variation.—The inescapable fact upon which statistical sampling plans are built is that there is inherent variation in the output of any manufacturing process. No two ball bearings have precisely the same diameter. No two capsules, tablets, or ampules will contain exactly the same amount of active ingredient. Even if it were possible to produce two identical units of product, we could never verify that they were exactly alike because there is no test procedure known to modern science which is capable of producing absolutely error-free measurements. The widespread belief that each tablet in a bottle contains exactly the same amount of active ingredient is every bit as erroneous as the notion that every male child in the United States will attain exactly the same weight at his fifth birthday. The difference is not one of kind—only of degree.

Hence, the problem of constructing an efficient acceptance sampling procedure is not simply one of determining whether or not each unit of product contains exactly the amount of active ingredient specified on the label. The problem is rather one of assuring that a "sufficiently large" percentage of the units falls "sufficiently close" to label claim.

Components of Variation.—In the discussion which follows, when we refer to the variation exhibited by a series of measurements we will be referring to *total* variation, unless otherwise specified. This total variation is not attributable to the product alone nor to the method of assay alone; it is actually the composite result of several variables simultaneously exerting their influences. For example, if a random sample of several tablets is drawn from a parent batch of a particular pharmaceutical product and an individual assay performed on each tablet, the observed variation among the resulting determinations represents the joint contribution of the following major factors: (*a*) differences in weight from tablet to tablet; (*b*) differences in the proportion of active ingredient from one tablet to another, resulting from inhomogeneous mixing of the drug with inert excipients in the granulation from which the tablets are compressed or molded; and (*c*) variation in assay, reflecting not only the precision of the measurement process but also such laboratory variables as sample preparation, equipment, and analysts.

In addition to the above, a lot-to-lot component of variation can reflect such additional factors as variation in raw material potency and variation in raw material assays.

A Typical Statistical Sampling Problem.—In order to illustrate the effectiveness of a statistical plan, together with the rationale behind it, let us take a realistic sampling problem and sketch the steps in its solution. Suppose an acceptance sampling procedure is required for finished lots of a particular tablet item. In other words, we wish to develop an efficient method for classifying a given production lot as "acceptable" or "unacceptable," basing this decision solely upon the information obtained from a small random sample of tablets drawn from the lot.

The first step is to arrive at explicit meanings for the terms "acceptable" and "unacceptable." If the product were an automobile tire, acceptable quality would be defined to reflect the mileage requirements of the consumer under typical driving conditions. If the product were an incandescent lamp, quality standards would incorporate the length of life that should be expected in normal usage. Similarly, quality requirements for an ethical drug should bear a close relationship to the therapeutic needs of the patient. If the range between the minimum effective dose and a toxic dose is small, tolerances on the product should tend to be narrow. On the other hand, if there is a broad dose range within which each dose is nontoxic and of equal therapeutic effect, the tolerances on the product should tend to be broad. By contrast, it appears that present official tolerances on drugs are frequently based, instead, upon the precision and accuracy that is attainable with current assay procedures. It may well be that more reasonable standards should reflect both factors.

A statistical sampling plan normally requires that four quality standards be specified: (*a*) an acceptable quality level (*AQL*), (*b*) an unacceptable quality level (*UQL*) (*c*) the risk of misclassifying an acceptable quality lot, sometimes referred to as the "Producer's Risk"(R_p), and (*d*) the risk of misclassifying an unacceptable quality lot, sometimes referred to as the "Consumer's Risk" (R_c).

When these four basic standards have been established, the corresponding sampling plan is completely determined. If the *AQL* and *UQL* are quite far apart and high risks of misclassification can be tolerated, as might well be the case with sodium chloride tablets, the plan will call for very few units in the sample. If the *AQL* and *UQL* are close together and very small risks of misclassification must be maintained, as on potent steroids, a large sample will normally be required.

In terms of our example, let us set up hypothetical quality standards that might be considered reasonable for a particular tablet product. We will assume that the unit of product from the point of view of the patient is a single tablet. Let us further assume that the definition of acceptable quality shown in Fig. 1 conforms to the therapeutic requirements for this item.

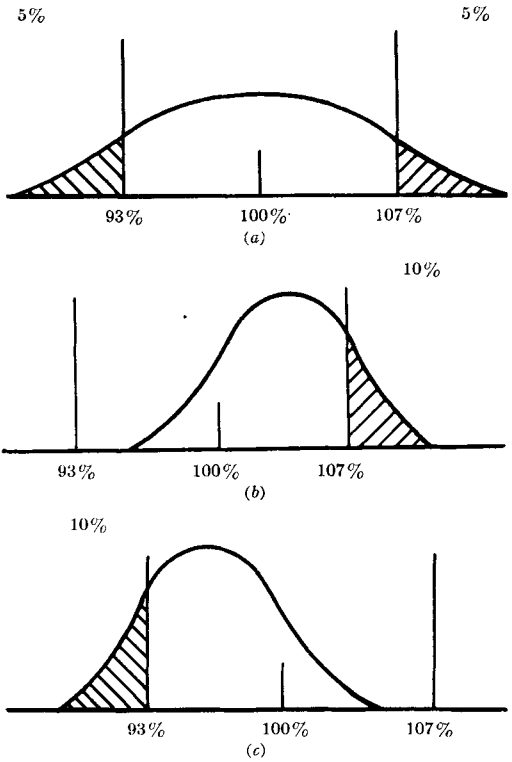


Fig. 1.—Distributions of single tablet assay values, acceptable quality, showing shifts in mean (*a*), mean at label claim; (*b*), upward shift; (*c*) downward shift.

A very large number of single tablet assay values should, when tabulated, distribute themselves under a bell-shaped curve in such a way that, for example, 90% of the tablets would fall within ±7% of label claim. This corresponds to an $AQL = 10\%$. In an analogous fashion let us define UQL as a lot of finished tablets with, say, 40% of its units outside of ±7% of label claim. Examples could be of the types illustrated in Fig. 2.

Let us assume further that we can tolerate misclassifying an acceptable lot no more than 10% of the time and an unacceptable lot no more than 10% of the time. We now have the four required standards: AQL, 10% of units outside ±7% of label claim; UQL, 40% of units outside ±7% of label claim; R_n, Producer's Risk = 10%; and R_c, Consumer's Risk = 10%.

It should be emphasized that we have chosen the numerical values for these standards quite arbitrarily for purposes of illustration. In actual practice this choice would, of course, rest with whatever official group was responsible for this type of policy decision.

Operating Characteristic Curve.—We have pointed out that R_p and R_c, the producer's and consumer's risks, are inherent in any sampling plan. A convenient graphical way of presenting these risks is through the operating characteristic (OC) curve.

In preparing to graph an OC curve, we take for the horizontal scale running from zero to 100%, the quality of submitted lots expressed as per cent defective, *i.e.*, the percentage of units in a lot which falls outside of the official tolerances for the product. On the vertical scale, also running from zero to 100%, is the percentage of lots of a given quality which is expected to be accepted if submitted to the sampling plan.

In an ideal situation, if we agree that a manufacturer is doing a good job if he submits to the sampling plan batches containing 10% defective units, we would accept all batches containing this proportion (or less) of defectives, and reject every batch containing 10.1% or more defectives (Fig. 3).

Such a plan would exhibit perfect discrimination. But it would be unrealistically stringent, for it would make no allowance for batch-to-batch variation about the 10% figure. What we want is a plan which will pass a high percentage of acceptable batches but at the same time guard against poor quality by accepting only a small proportion of bad lots if submitted. This would lead to a plan such as is shown in Fig. 4.

If we still concede that the manufacturer is doing a good job in submitting material containing 10% defective units ($AQL = 10\%$), we may want to be reasonably sure of passing this quality 95% of the time ($R_p = 5\%$). On the other hand, we may decide that 20% defective is intolerable ($UQL = 20\%$), and

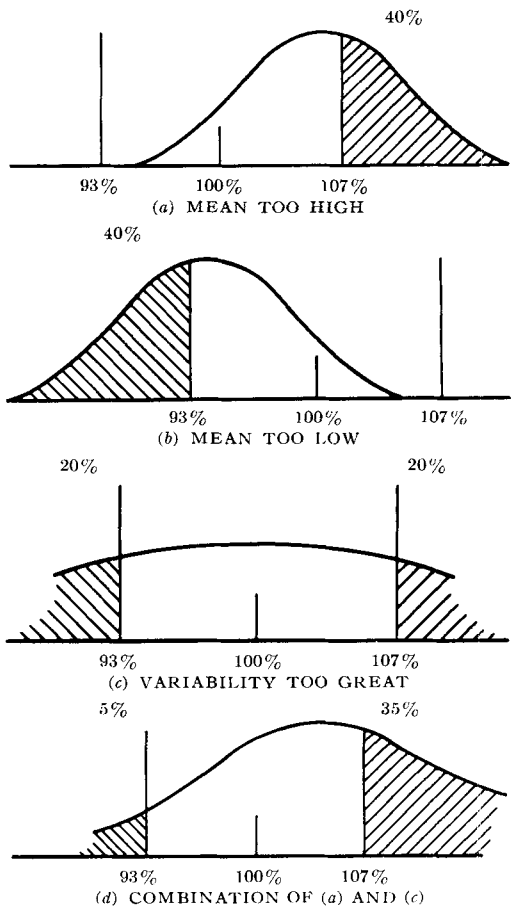


Fig. 2.—Distributions of single tablet assay values, unacceptable quality, showing shifts in location and dispersion.

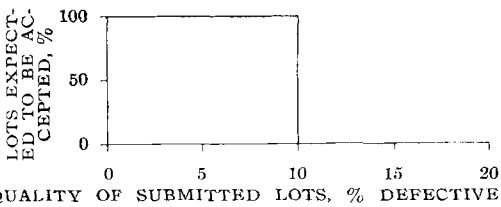


Fig. 3.—Ideal operating characteristic (*OC*) curve illustrating perfect discrimination but unrealistic stringency.

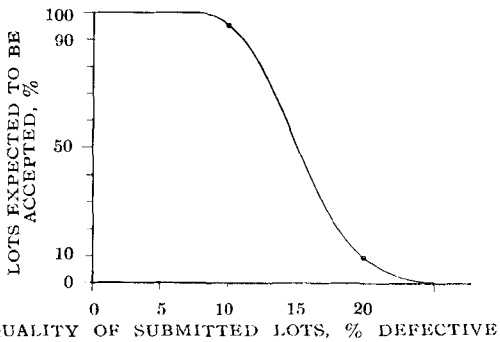


Fig. 4.—Typical *OC* curve from MIL-STD-414 (5), p. 27, illustrating $AQL = 10\%$; $UQL = 20\%$; $R_p = 5\%$; $R_c = 10\%$; $n = 85$.

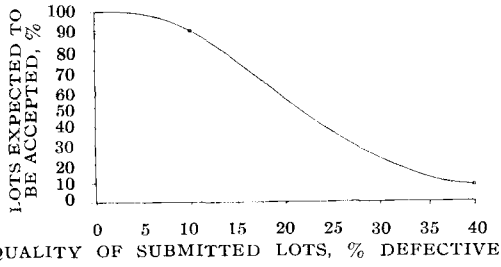


Fig. 5.—*OC* curve for tablet example, from MIL-STD-414 (5), p. 11, illustrating $AQL = 10\%$; $UQL = 40\%$; $R_p = 10\%$; $R_c = 8\%$; $n = 10$.

rarely want to pass this, certainly not more than 10% of the time ($R_c = 10\%$). Such a scheme, in practice, would be fair to both the manufacturer and to the patient.

Once these four characteristics of a plan have been agreed upon, the sample size is automatically determined. For the example cited, the sample size would be $n = 85$. Various *OC* curves for different values of sample size and per cent defective may be drawn, and judgment made as to properties of various sampling plans before agreeing upon one which balances the producer's and consumer's risks against the cost of assay. This balance has been discussed by Davies (4).

Construction of the Plan.—For single characteristics which may be measured on a continuous scale, and for which quality may be expressed in terms of per cent defective, the Military Standard 414 [MIL-STD 414 (5)] is an appropriate source of statistical plans if the measurements may be considered random, independent observations from a normal (bell-shaped) distribution. The mathematical and statistical principles underlying Military Standard 414 are described in a document of the same name (6). This reference also contains the specific methods used in computing the various tables and *OC* curves. In the case of pharmaceutical products we have defined per cent defective as that percentage of single dosage units which may be expected to fall above or below specified tolerances, such as those stated in the several individual monographs of the official compendia.

To illustrate the application of MIL-STD-414 (5), we refer again to our example for which tolerances were set at 93–107% of the labeled amount. Suppose this corresponds to 16.2 ±1.13 mg. of pure drug per tablet, *i.e.*, 15.07–17.33 mg./tablet. An inspection lot of tablets is presented for examination. We have previously specified $AQL = 10\%$; $UQL = 40\%$; $R_p = 10\%$; and $R_c = 10\%$. Searching through the operating characteristic curves of MIL-STD-414 (5) for the curve which most nearly corresponds to these requirements (Fig. 5), we find it associated with "Sample Size Code Letter F."

This *OC* curve tells us that lots of $AQL = 10\%$ (10% or less tablets outside of 15.07–17.33 mg./tablet) will be accepted 90% of the time (R_p). This is shown in Fig. 5 where the curve crosses the 10% abscissa at the 90% ordinate. We also see that lots of $UQL = 40\%$ will be accepted only 8% (R_c) of the time, *i.e.*, where the curve crosses the 40% abscissa at the 8% ordinate.

Referring to the appropriate master table for double specification limits, variability unknown, in MIL-STD-414 (5), we find the sample size corresponding to Code Letter F to be $n = 10$, and that the "acceptability criterion," estimated per cent defective, which balances the risks is 21.06%.

The final statistical plan then takes the following form: Select 10 tablets at random from an inspection lot and individually assay each tablet. Estimate the per cent defective in the lot from these 10 determinations. If this estimate does not exceed 21.06% defective, accept the lot. Otherwise, reject it.

Suppose that we obtain the following values: 16.20, 16.18, 15.70, 15.80, 16.05, 15.54, 16.19, 16.40, 17.00, 15.90 mg./tablet.

The details of computation are spelled out in Appendix I. In this instance the total estimated per cent defective is zero and the lot would be passed. Incidentally, the lot would also be passed on the basis of this sample if $AQL = 1\%$; $UQL = 11\%$; $R_p = 10\%$; $R_c = 20\%$.

Current Sampling Practices.—Let us now compare the discriminating ability of the above procedure with that of the typical plan currently specified in the U.S.P. (1) for a tableted product. The latter stipulates that a single assay on a composite sample of n tablets shall fall within $\pm y\%$ of the declared value. We are not concerned here with whether or not the numerical values of n and y are chosen properly. Our purpose is rather to point out that even with ideal choices of n and y, a plan of this type cannot be as effective as a statistical plan. The detailed derivations for this section are given in Appendix II.

Recalling the basic (but arbitrary) requirements specified for our example, and choosing $n = 10$ as the sample size in order to obtain an unbiased com-

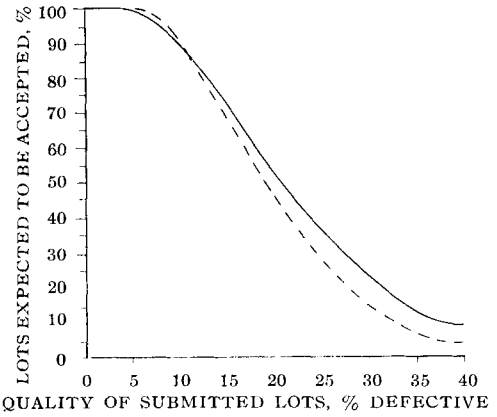


Fig. 6.—Comparison of *OC* curve for statistical plan (Fig. 5) with a curve computed for current plan (dashed line) when mean shifts.

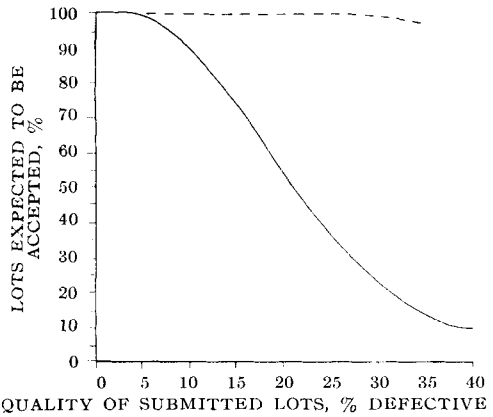


Fig. 7.—Comparison of *OC* curve for statistical plan (Fig. 5) with curve computed for current plan (dashed line) when variation becomes excessive.

parison with the statistical plan just derived, we find the ideal value for y to be approximately 5%. In other words, we have chosen the following specific plan: Perform a single assay on a composite sample of 10 randomly selected tablets and accept the parent lot only if the result falls within ±5% of the decleared value. The *OC* curve for this plan is shown in Fig. 6 for the situation in which inferior quality results from shifts in mean only. Note that this plan provides comparable discrimination to the statistical plan.

If the parent lot has mean potency low enough to force 10% of the tablets below 93% of label claim, both plans will pass this lot approximately 90% of the time. If 40% of the tablets in a lot fall below 93% of label claim because the mean potency of the lot is too low, the statistical plan will accept this type of lot about 8% of the time; the current type plan will accept it about 3% of the time.

Figure 7 shows the *OC* curve for the current plan when inferior quality results from increase in variation from tablet to tablet, the mean always at label claim.

It is clear that the plan is practically powerless to detect this type of trouble. For example, if 40% of the tablets in a lot fall outside ±7% of the declared value (and the lot mean is at 100%), this lot will be passed by the current plan about 94% of the time. The statistical plan, however, because it takes into account both mean and variation, will pass this lot only about 8% of the time, as before.

In actual practice one would expect unsatisfactory lots to exhibit some combination of simultaneous shift in mean and increase in variation. The power of the current plan varies considerably with the type of combination present. Two examples are shown in Fig. 8. The performance of the current plan is clearly inferior to the statistical plan for these combinations.

The reason for poor performance is clear. Because the current method relies on a single assay on a composite sample, it provides no way of estimating the variability in the amount of active ingredient from one tablet to another.

Recent Revisions.—The above shortcoming has been recognized; revisions in the official compendia have been recently proposed which take tablet-to-tablet variation into account. These proposals employ two separate criteria, one for detecting shift in mean only and another for detecting increase in variation only. Revisions of this type are certainly a step in the right direction, but they are still unable to compete with statistical plans because they cannot readily detect the unacceptable lot which has both difficulties operating simultaneously.

Using our example, a specific proposal might read as follows: Select 10 tablets at random from the inspection lot and accept the lot if the following two conditions hold. (*a*) the average drug content of the 10 individually assayed tablets falls within ±5% of the declared value, and (*b*) of the 10 results, not more than two shall differ from the average by more than ±7%.

Let us now examine the performance of this "2-step" plan relative to the statistical plan. If a lot of the quality shown in Fig. 9 is submitted, both the "2-step" plan and the statistical plan will accept it 90% of the time. Since this lot is, by definition,

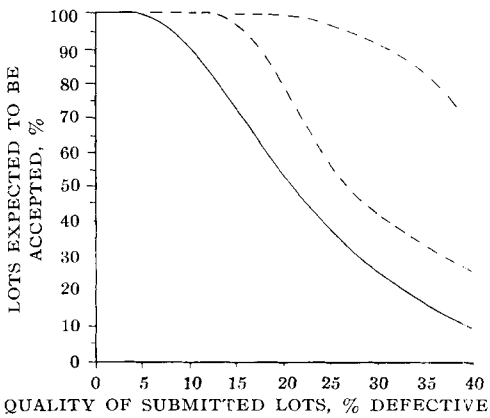


Fig. 8.—Comparison of *OC* curve for statistical plan (Fig. 5) with curves computed for proposed "2-step" plan (dashed line) for detecting shifts in (*a*) location and (*b*) dispersion.

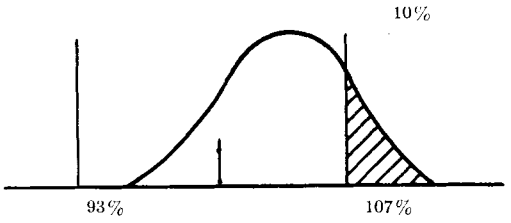


Fig. 9.—Distribution of single tablet assay values for lots expected to be accepted by both the statistical plan and proposed "2-step" plan. Upward shift in mean.

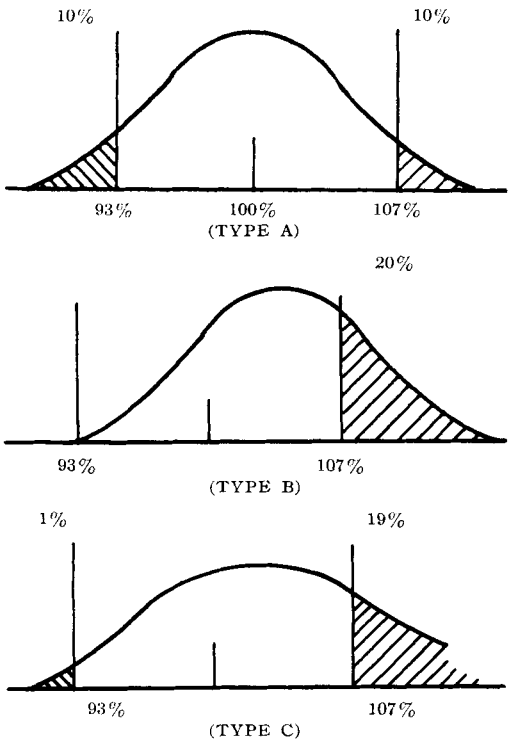


Fig. 10—Distribution of single tablet assay values for unacceptable quality lots of types shown in Table I. Type A, excessive variation; Type B, upward shift in mean; Type C, combination of excessive variation and upward shift in mean.

an acceptable quality lot, both plans are performing as desired. Next, let us consider three different types of lots, each having 20% of its tablets outside limits as illustrated by Fig. 10.

In Table I are shown the percentages of lots containing 20% defective tablets which we would expect to be accepted by each of the two plans, depending upon the type of distribution of the defective tablets outside the postulated official tolerances.

TABLE I.—PER CENT OF LOTS ACCEPTED

	Type A %	Type B %	Type C %
"2-Step" plan	74	50	86
Statistical plan	53	53	53

It is evident from Table I that the "2-step" plan is quite ineffective in detecting a lot of Type *C*, even though this lot has the same proportion of its tablets outside limits as do the other two. The shift in mean in lot *C* is not sufficient to be detected on this basis alone, the variation from tablet to tablet is not sufficient to be detected on this basis alone, and the plan cannot take into account the simultaneous operation of both. See Appendix III for details. By contrast, the statistical plan has the same effectiveness on all three lots.

Advantages of Statistical Plan.—It is clear from the preceding discussion that a statistically designed acceptance sampling plan has many advantages over a plan of any other type.

(*a*) It provides performance that is equal or superior to other plans using the same number of units of product in the sample. This is a result of efficient utilization of the information contained in the sample.

(*b*) Its performance can be studied in advance of its use, by examination of its operating characteristic curve. One can determine beforehand the quality levels that will be consistently accepted and those that will be consistently rejected, when stipulating appropriate risks. It aids in evaluation of official tolerances, not only from these standpoints, but also from that of practical economics in the determination of reasonable sample sizes.

(*c*) The statistical plan lends itself not only to the need of the manufacturer for sound acceptance sampling of his production lots, but also to that of the regulatory agencies who, having little information concerning in-plant quality controls, must perforce base their decisions on packages picked up in the market place. (It should be pointed out that such a package may not be expected to contain units which are randomly chosen from an entire production lot but will represent, rather, a small random sublot.)

The Contrasting Functions of Acceptance Sampling and Process Control.—We feel that this discussion would be incomplete without a word of caution regarding acceptance sampling in general. One characteristic common to all sampling programs is that a lot consisting of a large number of units is accepted or rejected on the basis of information obtained from a very small number of units. It is quite unreasonable to expect even the most efficient statistical plans to guarantee that practically all accepted lots will be of the desired quality. Although such a plan can be quite effective in detecting an occasional bad lot when all others are of high quality, it should be borne in mind that this is basically a checking device. A little reflection should convince even the most confirmed skeptic that an inspection operation which utilizes no prior information and which examines a very small percentage of the total output of a production process could easily overlook moderate departures from ideal quality a large percentage of the time. (For example, the statistical sampling plan discussed in this paper would accept lots containing 20% of their tablets outside tolerance limits about 53% of the time. If we wished to develop a plan which accepted lots of this quality only 1% of the time, it would require a prohibitively large number of units in the sample.) If a process were producing unacceptable quality consistently, acceptance sampling of the finished product would be singularly ineffective in preventing

TABLE II.—TABLET ACCEPTANCE PROTOCOL

Double specification limit.
Variability unknown.
Average range method.
One AQL value for both upper and lower specification limits combined.

Item: Tablets
Assay: ——————————
Label Claim: 16.2 mg./tab.
AQL: 10%

Tablet Assay Data: 16.20, 16.18, 15.70, 15.80, 16.05, R_1 = 16.20–15.70 = 0.50; 15.54, 16.19, 16.40, 17.00, 15.90, R_2 = 17.00–15.54 = 1.46.

Line	Information Needed	Value Obtained	Explanation
1	Sample size: n	10	
2	Sum of measurements: Σx	160.96	
3	Sample mean, $\bar{x} = \Sigma x/n$	16.096	160.96/10
4	Average range, $\bar{R} = \Sigma R$/no. subgroups	0.98	(0.50 + 1.46)/2
5	Factor c[1]	2.405	Table C-3
6	Upper specification limit: U	17.33	
7	Lower specification limit: L	15.07	
8	Quality index: $QU = (U - \bar{x})c/\bar{R}$	3.028	(17.33 − 16.096)2.405/0.98
9	Quality index: $QL = (\bar{x} - L)c/\bar{R}$	2.518	(16.096 − 15.07)2.405/0.98
10	Est. lot % def. > U: p_U	0.00	Table C-5
11	Est. lot % def. < L: p_L	0.00	Table C-5
12	Total est. % def. in Lot: $p = p_U + p_L$	0	
13	Max. allowable % of def.	21.06	
14	Acceptability criterion	$p < 21.06$	Accept. Lot

[1] For the derivation of Factor c, see pp. 23–24 of reference (6).

units of inferior quality from reaching the consumer.

A reputable pharmaceutical manufacturer must maintain production methods capable of sustaining the desired quality level and a quality control system which continuously monitors each process from raw material to finished product. This alone assures acceptable quality in the long run. The practice of sampling finished products serves as a check to insure that such a system is actually in use by detecting gross departures from desired quality if they occur.

APPENDIX I

Example of the Application of MIL-STD-414.—Table II shows a specimen protocol which includes details of computation of estimated per cent defective tablets in the lot from which were taken the tablets of our example. The particular situation deals with symmetrical double specification limits about the mean [(5) p. 69]. Other examples are given (5) where per cent defective beyond a single specification limit either above or below the mean may be estimated and also for double specification limits which are not symmetrical about the mean.

Since the procedure is spelled out in such detail, it may appear at first glance to be inordinately long. In practice, however, computation of quality indices and looking up the corresponding estimated per cent defective in Table C-5 (5) takes approximately 3 minutes.

Entries No. 1, 5, 6, 7, and 13 would normally be made in advance of the assay.

APPENDIX II

Operating Characteristic Curves for Current Plan.—The OC curves for the current plan were derived under the assumption that a sufficiently replicated assay of a composite sample of n tablets is equivalent to the average of n individual tablet assays. The mathematical formulation then takes the following form:

Let $x_1, x_2, \ldots, x_{10}$ be independently and identically distributed random variables, each having a normal distribution with mean μ and variance σ^2. Compute their arithmetic mean, $\bar{x}$. If $0.95\ \mu_0 < \bar{x} < 1.05\ \mu_0$, accept the parent lot; otherwise, reject it.

Let $P_A(\mu, \sigma)$ denote the probability of acceptance. Then we have

$$P_A = P_r\left\{0.95\,\mu_0 < \bar{x} < 1.05\,\mu_0\right\} = P_r\left\{\frac{0.95\,\mu_0 - \mu}{\sigma/\sqrt{10}} < u < \frac{1.05\,\mu_0 - \mu}{\sigma/\sqrt{10}}\right\} = F\left(\frac{1.05\,\mu_0 - \mu}{\sigma/\sqrt{10}}\right) - F\left(\frac{0.95\,\mu_0 - \mu}{\sigma/\sqrt{10}}\right) \quad \text{(Eq 1)}$$

where

$$u = \frac{\bar{x} - \mu}{\sigma/\sqrt{10}} \quad \text{and} \quad F(t) = \frac{1}{\sqrt{2\pi}}\int_{-\infty}^{t} e^{-y^2/2}\,dy$$

The per cent defective (% outside $\mu_0 \pm 0.07\ \mu_0$) which corresponds to $P_A\ (\mu, \sigma)$ can be expressed as

$$p(\mu, \sigma) = p_u + p_L$$

where

$$\begin{cases} p_u = \%\text{ above upper limit} = 1 - F\left(\dfrac{1.07\,\mu_0 - \mu}{\sigma}\right) \\ p_L = \%\text{ below lower limit} = F\left(\dfrac{0.93\,\mu_0 - \mu}{\sigma}\right) \end{cases}$$

The OC curve for shift in mean only (Fig. 6) was obtained by plotting $P_A(\mu, \sigma)$ *vs.* $p(\mu, \sigma)$ for various values of μ, keeping σ fixed at $\sigma = \sigma_0$. The curve for increase in variation only (Fig. 7) was obtained in analogous fashion by varying σ with μ fixed. Finally, the curves in Fig. 8 were obtained by simultaneous variation of μ and σ.

APPENDIX III

Performance Characteristics of Revised Plans.—The "2-step" plan described in *Recent Revisions* can be defined as follows:

Compute the arithmetic mean, $\bar{x}$, of the 10 sample values and accept the lot if the following conditions hold: (A) $0.95\ \mu_o < \bar{x} < 1.05\ \mu_o$, and (B) $|x_i - x| < 0.07\ x$ for at least 8 of the 10 samples values, x_i.

In the notation of the previous section, the probability that condition (A) holds is simply $P_A(\mu, \sigma)$, as given in Eq. 1. The probability, $P_B(\mu, \sigma)$ that condition (B) holds can be approximated by assuming that $x_i - \bar{x}$ and $x_j - \bar{x}$ are statistically independent when $i \neq j$. Letting $h = P_r\ [|x_i - \bar{x}_1| < 0.07\ \bar{x}]$ we obtain for P_B the binomial type expression

$$P_B(\mu, \sigma) = \sum_{r=8}^{10} \binom{10}{r} h^r (1-h)^{10-r}$$

where $h = 2F(0.0738\ \mu/\sigma) - 1$.

The value in Table I corresponding to a particular normal distribution was obtained by first computing μ, σ, $P_A(\mu, \sigma)$, $P_B(\mu, \sigma)$, and then approximating the probability of acceptance for the revised plan as $P'_A(\mu, \sigma) = P_A(\mu, \sigma)\, P_B(\mu, \sigma)$.

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Notes

The Occurrence of *iso*Pelletierine in *Withania somnifera*

By K. L. KHANNA, A. E. SCHWARTING, and J. M. BOBBITT

INVESTIGATION of the root alkaloids of *Withania somnifera* Dunal has resulted in the delineation of the alkaloid complex by paper partition chromatography (1), the isolation of tropine and pseudotropine (2), and the isolation and characterization of a new alkaloid, anaferine (3). Compound VII of the chromatogram, cited above, is *dl-iso*pelletierine. The occurrence of this alkaloid in the *Solanaceae* was first reported (4) for the leaves of *Duboisia myoporoides* R. Br.; the previously acknowledged occurrence being the root of *Punica granatum* L., family *Punicaceae*. This finding amplifies the known biochemical heterogeneity of the *Solanaceae* and extends a lysine related alkaloid to yet another genus in the plant kingdom.

EXPERIMENTAL [1]

The alkaloid was isolated from an ethanol extract of the defatted granulated root (81 Kg.). The concentrated extract diluted with water and adjusted to pH 4.7 was adsorbed on a column of Amberlite IRC-50-Na resin and was eluted by a gradient acid-buffer method. The fraction containing *iso*pelletierine hydrochloride was further purified by chromatography on acid alumina (Woelm, grade 1) using ethyl acetate as the eluent. The alkaloid salt (5.67 Gm.) was recrystallized from ethyl acetate.

***iso*Pelletierine Hydrochloride.**—The infrared spectrum was identical to that of a synthetic sample[2] and a natural sample;[3] m.p. 145°, undepressed in admixture with each of the above samples; $[\alpha]_D^{2.5} = 0.00$ (0.21% in ethanol).

Anal.—Calcd. for $C_8H_{16}ClN$: C, 54.07; H, 9.07; Cl, 19.95; N, 7.88. Found: C, 54.38; H, 9.59; Cl, 20.21; N, 7.67.

***iso*Pelletierine Picrate.**—The compound was prepared from the hydrochloride and was crystallized from ethanol; m.p. 148.5 to 149.5°, reported 149–150°. The melting point of a mixture with the picrate of an authentic sample[2] was undepressed.

***iso*Pelletierine 2,4-Dinitrophenylhydrazone Hydrochloride.**—The compound was prepared from the hydrochloride and was crystallized from ethanol-ethyl acetate; m.p. 240–241.5° (decompn.), lit. 242°.

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Received May 7, 1962, from the Pharmacognosy Research Laboratories, Pharmacy Research Institute and Department of Chemistry, University of Connecticut, Storrs.
Accepted for publication June 20, 1962.
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[1] Analysis by Geller Microanalytical Laboratories, Bardonia, N. Y. All melting points are corrected. Infrared spectra were determined in KBr pellets, using a Perkin-Elmer model 21 spectrophotometer.

[2] Supplied by Prof. J. B. Wibaut and Prof. H. O. Huisman, Holland.

[3] Supplied by Dr. P. I. Mortimer and Dr. J. W. Clark-Lewis, Australia.

APPENDIX III

Performance Characteristics of Revised Plans.—The "2-step" plan described in *Recent Revisions* can be defined as follows:

Compute the arithmetic mean, $\bar{x}$, of the 10 sample values and accept the lot if the following conditions hold: (A) $0.95\ \mu_o < \bar{x} < 1.05\ \mu_o$, and (B) $|x_i - x| < 0.07\ x$ for at least 8 of the 10 samples values, x_i.

In the notation of the previous section, the probability that condition (A) holds is simply $P_A(\mu, \sigma)$, as given in Eq. 1. The probability, $P_B(\mu, \sigma)$ that condition (B) holds can be approximated by assuming that $x_i - \bar{x}$ and $x_j - \bar{x}$ are statistically independent when $i \neq j$. Letting $h = P_r\ [|x_i - \bar{x}_1| < 0.07\ \bar{x}]$ we obtain for P_B the binomial type expression

$$P_B(\mu, \sigma) = \sum_{r=8}^{10} \binom{10}{r} h^r (1-h)^{10-r}$$

where $h = 2F(0.0738\ \mu/\sigma) - 1$.

The value in Table I corresponding to a particular normal distribution was obtained by first computing μ, σ, $P_A(\mu, \sigma)$, $P_B(\mu, \sigma)$, and then approximating the probability of acceptance for the revised plan as $P'_A(\mu, \sigma) = P_A(\mu, \sigma)\, P_B(\mu, \sigma)$.

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Notes

The Occurrence of *iso*Pelletierine in *Withania somnifera*

By K. L. KHANNA, A. E. SCHWARTING, and J. M. BOBBITT

INVESTIGATION of the root alkaloids of *Withania somnifera* Dunal has resulted in the delineation of the alkaloid complex by paper partition chromatography (1), the isolation of tropine and pseudotropine (2), and the isolation and characterization of a new alkaloid, anaferine (3). Compound VII of the chromatogram, cited above, is *dl-iso*pelletierine. The occurrence of this alkaloid in the *Solanaceae* was first reported (4) for the leaves of *Duboisia myoporoides* R. Br.; the previously acknowledged occurrence being the root of *Punica granatum* L., family *Punicaceae*. This finding amplifies the known biochemical heterogeneity of the *Solanaceae* and extends a lysine related alkaloid to yet another genus in the plant kingdom.

EXPERIMENTAL [1]

The alkaloid was isolated from an ethanol extract of the defatted granulated root (81 Kg.). The concentrated extract diluted with water and adjusted to pH 4.7 was adsorbed on a column of Amberlite IRC-50-Na resin and was eluted by a gradient acid-buffer method. The fraction containing *iso*pelletierine hydrochloride was further purified by chromatography on acid alumina (Woelm, grade 1) using ethyl acetate as the eluent. The alkaloid salt (5.67 Gm.) was recrystallized from ethyl acetate.

***iso*Pelletierine Hydrochloride.**—The infrared spectrum was identical to that of a synthetic sample[2] and a natural sample;[3] m.p. 145°, undepressed in admixture with each of the above samples; $[\alpha]_D^{2.5} = 0.00$ (0.21% in ethanol).

Anal.—Calcd. for $C_8H_{16}ClN$: C, 54.07; H, 9.07; Cl, 19.95; N, 7.88. Found: C, 54.38; H, 9.59; Cl, 20.21; N, 7.67.

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Quantitative Determination of Dehydrocholic Acid in Tablets

By VISHNU DAS GUPTA

Quantitative recovery of dehydrocholic acid from the tablets can be accomplished by first extraction with chloroform and subsequent titration as given in the U.S.P. XVI. Results are reported to indicate that the residue obtained is of pure acid.

THE U.S.P. XVI (1) procedure for the estimation of dehydrocholic acid appears to be satisfactory for pure samples of acid but reproducible results are obtained only with difficulty for some of the commercial preparations. Some samples require stirring for periods up to 1 hour on the magnetic stirrer before solutions are effected in order to obtain quantitative recoveries; without stirring there is a marked tendency to obtain premature end points which are difficult to distinguish from the true end point. The addition of 100 ml. of water as prescribed shortly before the end point is reached may also lead to low results if added too soon. This is probably caused by some of the acid going out of solution. Crisafio and Chatten (2) of the National Food and Drug Laboratories, Ottawa (Ontario) have reported similar difficulties.

EXPERIMENTAL

Not less than ten tablets were reduced to a fine powder and a portion of the powder, representing about 300 mg. of dehydrocholic acid was accurately weighed and transferred to a dry 100-ml. volumetric flask with the aid of reagent grade chloroform. Sufficient chloroform was added to make exactly 100 ml. of the mixture and the flask was agitated. The mixture was allowed to stand for 1 minute and rapidly filtered through dry rapid filter paper. The first 20 ml. was rejected and the next 50 ml. of the filtrate was collected in a dry 50-ml. volumetric flask which was transferred to a 250-ml. Erlenmeyer flask or a 100-ml. tared beaker if the residue was to be weighed. The flask was washed with two 5-ml. portions of chloroform and mixed with the filtrate. Chloroform was removed by evaporation; 2 ml. of dehydrated alcohol was then added and also removed by evaporation. The residue was cooled and assayed by U.S.P. method (1) for dehydrocholic acid, using 0.02 *N* sodium hydroxide. Each milliliter of 0.02 *N* sodium hydroxide is equivalent to 8.05 mg of anhydrous dehydrocholic acid. If the tablets were known to contain no chloroform-soluble excipients such as stearic acid, then the residue was dried at 100° for 15 minutes, cooled in a desiccator, and weighed. Twice the weight of the residue represented the amount of dehydrocholic acid contained in the portion of the powder taken. The melting point of the residue and the U.S.P. method (1) for determining the dehydrocholic acid confirmed that the residue was a pure form of the dehydrocholic acid. Results are recorded in Table I.

SUMMARY AND CONCLUSION

1. A study was designed to develop a method for 100% recovery of dehydrocholic acid from the tablets.

2. The method described required 20 minutes (actual working time) for each analysis with 100% recovery. The solvent used is available in every laboratory.

3. Assay results appear to confirm the accuracy of the method.

TABLE I.—ASSAY RESULTS FROM DEHYDROCHOLIC ACID TABLETS

Sample No.	Recovery, U.S.P. Method	Recovery, Proposed Method	M.p. of Residue	Percentage Found in the Residue by U.S.P. Method
1	100.5	102.2	238–241°	102.2
	100.3	102.3	as above	102.1
	100.3	102.5	as above	102.2
2	98.1	100.5	as above	100.1
	98.0	100.5	as above	100.1
	97.7	100.7	as above	100.2
3	99.2	101.2	as above	100.9
	99.5	101.5	as above	101.4
	99.7	101.7	as above	101.4
4	103.7	105.9	as above	105.4
	102.5	105.8	as above	105.4
	102.8	105.8	as above	105.4
5	97.4	99.7	as above	99.5
	97.8	99.9	as above	99.5
	97.3	99.2	as above	99.0
6	98.2	100.1	as above	99.8
	97.5	100.3	as above	99.9
	97.2	100.3	as above	99.8

Received May 10, 1962 from the Quality Control Division, Schlicksup Drug Company, Inc., 420–22 S.W. Washington St., Peoria, Ill.

Accepted for publication May 25, 1962.

The author wishes to thank Dr. Frederick V. Lofgren, Professor of Pharmacy at the University of Texas, for his invaluable suggestions in the writing of this paper.

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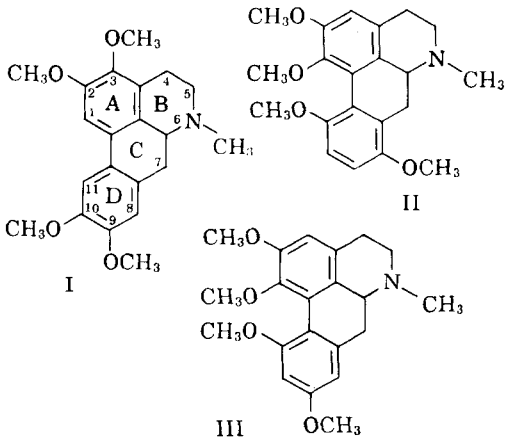
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Observations on the Structure of Argemonine

Sir:

Our interest in certain of the *Argemone* alkaloids (1–3) recently led us to postulate three tentative aporphine structures for argemonine (4). All of these structures adopted a 2:3 placement for two of the methoxyl groups with the other two vicinally placed in the 8:9, 9:10, or 10:11 positions. The 9:10 position was favored, largely on the basis of negative oxidative evidence that had failed to indicate the presence of isolable *o*-hemipinic acid (3,4-dimethoxyphthalic acid) in the oxidation residues with only *m*-hemipinic acid (4,5-dimethoxyphthalic acid) being observed. Thus, structure I was suggested as being the most reasonable structure for argemonine with the evidence in hand at that time. Since then, Shamma (5) has postulated two additional structural possibilities (II and III) in an attempt to reconcile our low ultraviolet extinction coefficients for argemonine and its related alkaloids. His reasoning was that 1:11 methoxyl interaction would accentuate the known twist of the biphenyl moiety in the aporphine nucleus, thereby reducing coplanarity with resultant reduction in absorption. On this basis, he found it necessary to adopt a *non-vicinal* arrangement of methoxyls in ring *D* to implement his arguments. His reasoning for a 1:2 placement of the ring *A* methoxyls was based on the observation (6) that all known naturally-occurring aporphine alkaloids have the 1:2 arrangement. Our recent oxidative experiments have shown that Shamma's assumptions are unfounded.



We have ethylated the monophenol, norargemonine[1], with diazoethane and oxidized the resultant ethylnorargemonine without further purification, other than removal of unreacted norargemonine, with manganese dioxide in dilute sulfuric acid by the same procedure as we had previously oxidized argemonine (4). The neutral fraction from this oxidation yielded a small amount of N-methyl-*m*-hemipinimide, m.p. 261–262°, identified by mixed melting point with authentic material prepared by the usual method from authentic *m*-hemipinic acid. The acidic fraction from the above oxidation, on being treated with ethylamine and subsequently heated to 180° and sublimed, yielded N-ethyl-4-ethoxy-5-methoxyphthalimide, m.p. 202–203°. The identity of this product was established by mixed melting point determination and comparison of X-ray powder diffraction patterns, ultraviolet, and infrared spectra with the authentic compound prepared by permanganate oxidation of boldine that had been completely ethylated with diazoethane, followed by the usual treatment to obtain the desired phthalimide derivative. The above findings, assuming an aporphine ring system, show that the phenolic group in norargemonine is on ring *D* rather than on ring *A* and, therefore, indicate that the placement of the methoxyl groups in ring *D* of argemonine is *vicinal* and must be the same as that found in glaucine, namely 9:10. Ring *A* obviously could bear the methoxyls in the 1:2 or 2:3 positions because the necessary *o*-decarboxylation to yield N-methyl-*m*-hemipinimide does not indicate the placement unequivocally but could have arisen from either arrangement. The 1:2 placement, however, could not be entertained as a possible structure because this is the arrangement in glaucine, an isomer of argemonine, which is not identical with the latter alkaloid. Thus, structure I is the only remaining possibility for argemonine.

We previously had referred (4) to the recent elucidation of the structure of crebanine (9) in which a biogenetically unacceptable 8:9 placement of vicinal methoxyls was found in ring *D* and a normal 1:2 placement in ring *A*. If our structural arguments are valid, argemonine, on the other hand, represents a biogenetically acceptable 9:10 placement in ring *D* but an abnor-

[1] Manske and Ashford (7) have suggested that norargemonine "could be partly racemic isocorypalmine." Our own experience in methylating it to argemonine and the similar findings of Boit and Flentje (8) indicate that the above statement is without basis and that norargemonine can be considered a homogeneous entity related to argemonine as a monophenol to its methyl ether.

mal 2:3 placement in ring A. To our knowledge, this would be the first example of such an aporphine alkaloid and, together with the crebanine structure, illustrates the necessity for extreme caution when applying biogenetic principles to solving structural problems in the aporphine group.

We are carrying out presently the synthesis of argemonine to further implement our structural assignment. This work will be the subject of a future communication.

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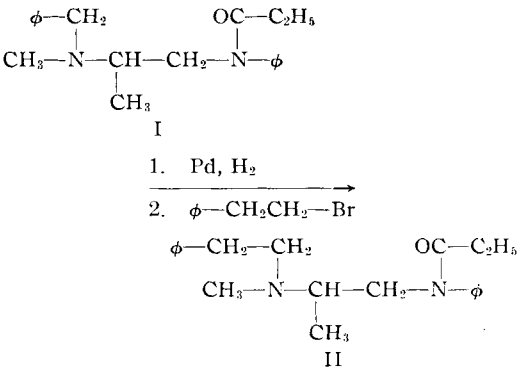
Received September 26, 1962.
Accepted for publication October 9, 1962.
We acknowledge with gratitude the assistance of Dr. Tibor Z. Zoltai, Department of Geology, University of Minnesota in carrying out the X-ray diffraction comparisons and of Dr. Michael J. Martell, Jr., Lederle Labs., Pearl River, New York, for arranging to have infrared and ultraviolet spectra run for us by his firm.

The Absolute Antipodal Activity of Analgesics in the Basic Anilide Series

Sir:

The influence of absolute configuration on the activity of potent analgesics possessing one asymmetric center has been demonstrated by Beckett and co-workers (1). These experiments have shown that most of the analgesic activity resides in the enantiomorph possessing the D-configuration. A hypothesis was advanced which attempted to define an analgesic receptor surface on the basis of three points of contact. This implies that the orientation of the methyl group on the asymmetric carbon of the methadones and thiambutenes is held in a favorable position only in those compounds having the D-configuration. In an effort directed at testing this hypothesis we have determined the absolute configuration of diampromid (II) (2), a member of a new class of analgesics of the basic anilide type.

The D-(−)-N-methyl-N-benzyl compound (I) (3), which has been related to D-alanine, was catalytically hydrogenolyzed with palladium on carbon according to the procedure of Wright, Brabander, and Hardy (2). The resulting debenzylated intermediate was then phenethylated with phenethyl bromide to give D-(−)-diampromid (II), $[\alpha]_D^{28°}$ −26.4° (c 5% in ethanol), b.p. 159–163° (0.1 mm.). The infrared spectrum is identical with that of racemic II (2). Wright, Brabander, and Hardy (4) have independently prepared (+) and (−)-diampromid. Their physical constants are in close agreement with our data.



The (+) and (−) enantiomers of compound I and of diampromid (II) were tested (5) subcutaneously on rats by a modification of the rat-tail heat response method. The L-(+)-isomers of I and II possess analgesic activity comparable to that of morphine, whereas the D-(−)-isomers are substantially less active. These pharmacological results are quite unexpected since it has been demonstrated that the more active enantiomorphs of methadone-type and thiambutene-type analgesics have the D-configuration (1). The potent analgesic action and addictive properties of I and II suggest that they are acting by a mechanism similar to that of methadone and other potent analgesics in spite of the fact that the more active enantiomorphs

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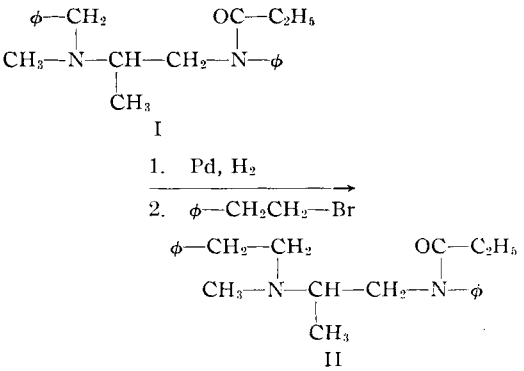
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of these compounds are related to L-(+)-alanine. The apparent inversion of antipodal activity on passing from the methadones to the basic anilides suggests that in addition to the orientation of the methyl group on the asymmetric carbon of I and II, other factors may also exert a profound influence on the absolute antipodal activity of the basic anilides.

We are presently investigating the configurations of other basic anilide analgesics and methadone-type compounds.

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Received September 6, 1962.
Accepted for publication October 1, 1962.
The author gratefully acknowledges the cooperation of Dr. Robert A. Hardy, Jr., of Lederle Laboratories, in supplying physical data for comparison purposes, and in being instrumental in informing us of the pharmacological results.

Book Notices

Methods of Biochemical Analysis. Vol. 10. Edited by DAVID GLICK. John Wiley & Sons, Inc., Interscience Division, 440 Park Ave. South, New York 16, N. Y., 1962 xi + 399 pp. 15 × 23 cm. Price $14.50.

This tenth volume in a series concerned with methods of biochemical analysis contains sections on Separation of carbohydrate derivatives by gas-liquid partition chromatography, Determination of-SH groups in proteins, Measurement of sodium and potassium by glass electrodes, Measurement of UDP-Enzyme systems, Determination of dissociation constants for two-substrate enzyme systems, Dialysis, Countercurrent distribution, Partition methods for fractionation of cell particles and macromolecules, Determination and microscopic localization of cholesterol, and Chemical determination of flavins. Subject and author indexes for this volume as well as a cumulative index for the entire series enhance the value of the series as a reference source. Numerous literature references follow each section which should be an aid to workers employing the methods and techniques described.

Plante Medicinali, Chimica Farmacologia e Terrapia. Vol. 1. By BENIGNI C. CAPRA, and P. E. CATTORINI. dott. Inverni & Bella Beffa S.p.a., Via Ripamonti n. 99, Milano, Italy, 1962. vi + 730 pp. 16 × 23 cm. Price L. 8000.

This is a reference work (in Italian) on medicinal plants which covers chemical, pharmacologic, and therapeutic aspects of these substances. Volume 1 is apparently the first of a series since this volume includes plant names in the A-G group. The book is arranged alphabetically by plant name in monograph form. Each monograph includes identifying characteristics, extracts, and usual preparations with formulas as well as good, frequently extensive, literature references. An index is appended.

Analyze des Stéroïdes Hormonaux. Méthodes générales. Tome 1. Vol. 1. By M. F. JAYLE. Masson et Cie Editeurs, 120 boulevard Saint-Germain, Paris VI e, France, 1961. 276 pp. 16.5 × 24.5 cm. Price 45 NF.

This volume (in French) is one of a three volume set dealing with steroids and hormones. Volume 1 concerns itself with the structure, nomenclature, physical constants, hydrolysis, extraction, and purification of steroids. It also covers the analysis of conjugated steroids and spectophotometric and chromatographic analysis of steroids.

Précis de Botanique. Tome 1. Vol. 1. Morphologie et Reproduction des Plantes Vascularies, Systematique des Cryptogames, Vascularies et des Gymnospermes. By P. CRÉTÉ. Masson et Cie, Editeurs, 120 boulevard Saint-Germain, Paris VIe, France, 1962. viii + 348 pp. 16.5 × 21.7 cm. Price 34 NF.

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